The Hsp90 Requirement in the Slt2p Stress-Activated MAP kinase Activity of Yeast

Andrew Truman

Thesis submitted to University College London for the degree of Doctor of Philosophy

Department of Biochemistry and Molecular Biology
UCL
Gower Street
London WC1E 6BT

March 2005

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ABSTRACT

The Hsp90 molecular chaperone function is essential and extremely well conserved in eukaryotic organisms, yet much of the mechanism of Hsp90 action remains a mystery. This investigation set out to identify a model client protein of Hsp90 in the yeast *Saccharomyces cerevisiae* that would be amenable to structural and genetic analysis.

Initial studies were conducted on *HSF (1-583)* strain, which has a C-terminally truncated heat shock transcription factor resulting in low Hsp90 expression. This mutant displayed a swollen cell phenotype that could be suppressed with osmotic stabilisation, indicating that the mutant had defective maintenance of cell wall integrity under stress conditions. This was shown to be a result of an inability to activate Rlm1p, the transcription factor regulated by the cell integrity MAP kinase pathway.

Expression of the T22I mutant form of the Hsp82 isoform of Hsp90 expressed as the sole Hsp90 of the cell was also shown to generate a cell integrity defect. Unlike with *HSF (1-583)*, this is apparent at low as well as high temperatures of growth. An interaction between Hsp82 and the MAP kinase Slt2p, the activator of Rlm1p, was demonstrated by both protein binding and two hybrid studies. This interaction was shown to be reinforced under cell wall stressing conditions and dependent on the phosphorylation status of Slt2p.

The final part of this investigation revealed that mammalian BMK1 (ERK5) MAP kinase can substantially provide Slt2p MAP kinase function in yeast. We demonstrated that this BMK1 protein could associate with both isoforms of Hsp90 in yeast, Hsp82 and Hsc82.

Overall, the data indicated the Slt2p MAP kinase is a client protein of Hsp82, providing the first evidence for an Hsp90 involvement in activity of a member of the MAP kinase family proteins.

ACKNOWLEDGEMENTS

"Two things are infinite: the universe and human stupidity; and I'm not sure about the universe."

-Albert Einstein

"You need to keep an open mind, but not so open your brain falls out"

-Dr. Frank Cooke

*

I would like to thank my supervisor, Prof. Peter Piper for his guidance and sharing his great expertise in the field of yeast genetics and molecular biology with me. Thanks must go out to members of the lab who have persevered with me for these years, namely Nick Harris (whose patience in teaching me to setup western blot equipment was nothing short of heroic); Kostas Hatzixanthis ('LacZ king' and who passed on his expertise on the subject); Stefan Millson (Who has taught me everything from PCR to the yeast two-hybrid system); Mehdi Mollapour (for introducing me to the world of GFP microscopy) and Vicky King who has put up with my endless chatter for a good one and a half years without going insane.

Everyone says they remember a good teacher, and I would like to thank the exceptional skills of Leslie Thomas who first recognised my interest in the life sciences and encouraged me to continue on with them.

Finally thanks must go to my parents and my brother Steven who has supported and encouraged me through these years.

ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
bp	base pair
cAMP	cyclic adenosine monophosphate
d	day
dATP	adenosine deoxyribonucleoside triphosphate
dCTP	cytosine deoxyribonucleoside triphosphate
dGTP	guanosine deoxyribonucleoside triphosphate
dNTP	equimolar mixture of dATP, dCTP, dGTP dTTP
dTTP	thymine deoxyribonucleoside triphosphate
dH_2O	distilled water
DTT	dithiothreiol
EC	endothelial cell
ER	endoplasmic reticulum
GDP	guanosine diphosphate
GFP	green fluorescent protein
GTP	guanosine triphosphate
HSE	heat shock element
HSF	heat shock factor
Hsp	heat shock protein
IMAC	immobilized metal ion affinity chromatography
OD	optical density
ORF	open reading frame
SD	standard deviation
SDS	sodium dodecyl sulphate
Y2H	yeast two-hybrid

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1 Introduction

Whenever a cell is exposed to a stress, such as heat shock, it must provide measures to reduce the detrimental effects of this stress on its internal structures and its homoeostatic status. One of the counter-measures produced by heat shock is the strong induction of a small number of protective proteins known as the heat shock proteins (Hsps) (Morimoto, 1991). Many Hsps are molecular chaperones whose general function is to assist in protein folding and the assembly of protein structures, but also to aid in the recovery of the cell from stress induced protein damage.

One of the least understood of these chaperone functions is that of Hsp90. An essential protein in eukaryotic cells (Borkovich et al., 1989), cytosolic Hsp90 is expressed at relatively high levels (1-2% of total cell protein) even in the absence of stress (Borkovich et al., 1989). Unlike certain other well- characterised chaperones such as Hsp70, Hsp90 does not play a part in the *de novo* folding of most proteins. Instead it seems to be needed for the activation step of several important signalling and regulatory proteins, the Hsp90 'clients'. Hsp90 acts on the latter when they are already substantially folded (often after the Hsp70 family proteins have acted on them), so as to enable the final structural changes that enable attainment of the active conformation. *In vitro*, Hsp90 can slow the aggregation of denatured proteins (Wiech et al., 1992), but it is not clear whether this activity has any relevance to its *in vivo* function.

Two significant discoveries have boosted recent interest in the Hsp90 field. Firstly, it has been found that Hsp90 is a key factor determining whether the widespread variation affecting developmental pathways in the genotypes of natural populations is expressed phenotypically. This variability is largely silent except under conditions of stress. Hsp90 buffers this variation, thereby allowing it to accumulate under neutral conditions. When development occurs under slightly supraoptimal temperatures or in the presence of low levels of Hsp90 inhibitor drugs, Hsp90 buffering is compromised thereby allowing otherwise cryptic mutants to be expressed. In this way, Hsp90 allows stress to induce

phenotypic changes in otherwise rooted developmental processes (Ikeda et al., 1997). Secondly, it has been found that Hsp90 is a promising anticancer drug target since Hsp90 is needed for the activity of many important oncoproteins (such as p53, Kit, Flt3 and v-src). Hsp90 inhibition can therefore inactivate simultaneously several of the activities responsible for multistep oncogenesis. Phase two clinical trails are already in progress for one inhibitor of Hsp90, 17-AAG (a derivative of the natural antibiotic geldanamycin (Workman and Maloney, 2002)). Furthermore, fragments of Hsp90 protein extracted from malignant tumours can be re-injected to the suffering organism in the form of a vaccine (Srivastava and Amato, 2001). In many cases, the tumour significantly decreases in size.

Many approaches have been used to study cytosolic Hsp90, enveloping studies of the single Hsp90 of *Drosophila melanogaster*; the two major isoforms of Hsp90 in humans; to the seven versions of Hsp90 in *Arabidopsis thalania*. These organisms have all contributed much to the Hsp90 field, but for genetic analysis of Hsp90 function, the budding yeast *Saccharomyces cerevisiae* has many advantages. The two isoforms of cytosolic Hsp90 in yeast, Hsp82 and Hsc82 have 50-60% sequence identity with the human Hsp90 isoforms, and expression of these human Hsp90s is able to provide most Hsp90 functions in yeast ((Picard, 2002) ((Piper et al., 2003b)). In addition yeast readily lends itself to genetic manipulations that are more difficult to perform in other organisms. However, yeast does not have the versions of Hsp90 that are found in the endoplasmic reticulum (Grp94) or the mitochondrion (TRAP-1) of mammalian cells, thus it is really only suited to the study of cytosolic forms of Hsp90.

1.1 The heat shock response

The heat shock gene expression program is extremely well conserved, its main goal being to increase Hsp expression as rapidly as possible. In eukaryotic organisms, heat shock transcription factor (HSF) directs this heat shock response. HSF binds to a characteristic DNA element consisting of alternating repeats of the 5bp sequence, nGAAn (the heat shock element (HSE)). In metazoans it is the trimerisation and nuclear

translocation of HSF in response to disulphide bond formation (Ahn and Thiele, 2003), (leading to binding of the trimerised HSF to the HSEs in the promoters of target genes) that results in transcription of these gene in response to heat shock. In yeast, the HSF is constitutively trimerised and much of it is constitutively bound to HSEs in the absence of stress ((Sorger and Pelham, 1988), (Hahn and Thiele, 2004)). Yeasts have just a single HSF (Hsf1p), whereas up to four different HSFs can co-exist in plant, avian and mammalian cells (Voellmy, 2004). Yeast Hsf1p potently activates Hsp gene transcription under heat shock stress, leading to a rapid increase in heat shock mRNA levels that peaks at around 15 minutes after temperature upshift. Hsf1p is an essential protein in budding yeast and its mutation can result in decreased expression of some proteins even at non-heat shock temperatures (i.e. Hsp82, Sti1p and Cpr6p (Nicolet and Craig, 1991)). It appears that increased activity under heat shock conditions comes from an increased occupancy for specific HSE's ((Erkine et al., 1999) (Tamai et al., 1994)). Selectivity can also be achieved by the pattern of HSE repeats in target gene promoters. Thus the *CUP1* promoter sequence contains an imperfect match to the classic triple repeat of the HSE that includes a perfectly spaced gap, resulting in undetectable *CUP1* gene expression at 37°C, but a strong induction at 39°C (Santoro et al., 1998).

In contrast to the HSF of metazoans that has just a single activation domain, yeast HSF has two independent transcriptional activation domains. In the ensuing discussion these are referred to as the amino-terminal transcriptional activation domain (NTA; residues 1 to 172) and carboxyl-terminal transcriptional activation domain (CTA; residues 584 to 833). Derepression of these domains seems to be the main mechanism of activating HSF. The specific requirement for CTA domain-directed gene expression at high temperatures appears to be induction of a high level of Hsp82, the strongly heat-inducible isoform of Hsp90 (Morano et al., 1999). This thesis describes experiments that show that this high Hsp82 level is required in order to facilitate the activation (via Slt2p MAP kinase) of the reinforced cell integrity gene expression needed for yeast growth at high temperatures (Chapters 3 and 4).

One other potential control over HSF is phosphorylation. Yeast Hsf1p (93.2kDa) migrates at an apparent size on SDS gels of between 150-160kDa when isolated from cells grown at 30°C and up to 190kDa when isolated from heat shocked cells. This is due to altered phosphorylation state (Hoj and Jakobsen, 1994). Deletion of a short sequence in Hsf1p implicated in the regulation of transcription (control element 2) results in an increased phosphorylation in the absence of heat stress, suggesting an inhibitory role as well as an activatory role for the phosphorylation of Hsf1p (Hoj and Jakobsen, 1994). In addition, it has recently been shown that HSF is a substrate for phosphorylation by Snf1p protein kinase *in vitro*, suggesting a role for Hsf1p in the global response to glucose starvation (Hahn and Thiele, 2004). Chromatin experiments indicate some 3% of yeast genes may be subject to HSF regulation (Hahn et al., 2004).

Mammalian HSF1 senses stress directly through intramolecular disulphide bond formation (Ahn and Thiele, 2003). However it remains a mystery how yeast Hsf1p senses heat stress and triggers the heat shock response, as Hsf1p lacks cysteine residues. Many have investigated a possible interrelationship of yeast HSF activation and the cell integrity MAP kinase cascade, simply because both are activated by heat stress. However, studies of activity of an HSE-*LacZ* reporter gene in various cell integrity mutants have failed to demonstrate any cell integrity MAP kinase pathway control over HSF activity ((Kamada et al., 1995) (Zu et al., 2001)). This thesis reveals (Chapter 3) that the control is actually the opposite way round, that Hsf1p (indirectly through its effects on Hsp82 levels) controls activation of genes by the cell integrity MAP kinase pathway.

Besides increased activity of HSF elevating Hsp90 levels (and therefore presumably, Hsp90 activity), there is evidence that the Hsp90 system also appears to deactivate HSF. A double mutant strain lacking both Hsp82 and the Cyp-40 homologue Cpr6, exhibits derepression (greater than 30-fold) of Hsf1p transcriptional activity in the absence of stress, as measured through HSE-*LacZ* reporter activity (Duina et al., 1998). Inhibition of Hsp90 function, either by mutation (Harris et al., 2001) or by compounds such as geldanamycin results in increased levels of HSE-*LacZ* activity (Piper et al., 2003a), also

increases HSE-*LacZ* expression. In mammalian cells Hsp70 levels are important in HSF1 regulation (Voellmy, 2004), but in yeast this does not appear to be the case. Overexpression of the Ssa2p cytosolic Hsp70 fails to repress HSF activation in a stressed cell, while a constitutively activated mutant HSF (R451A) led to increased levels of molecular chaperones, but no alteration to the HSF temperature activation setpoint (Hjorth-Sorensen et al., 2001). Overexpression of Hsp82 from a high-copy-number vector also does not alter the heat shock induction of Hsp104, Hsp70, Hsp35 or Hsp26 and has little effect on thermotolerance (Cheng et al., 1992).

Lee and co-workers (Lee et al., 2000) observed that purified *S.cerevisiae* Hsf1p can be induced to form an altered conformation in response to agents that induce HSF activity *in vivo*: heat and oxidation. This indicates that yeast HSF, like the mammalian HSF1 is sensing these stress agents directly, even though it lacks the redox-active disulphides of the latter (Ahn and Thiele, 2003).

1.2 Structural elements of the Hsp90 molecular chaperone

The current studies on Hsp90 appear to be directed to two main areas, firstly, the search for new Hsp90 client proteins and secondly, the continuation of structural studies on Hsp90 in complex with its co-chaperones. Both types of study will increase our understanding of what enables Hsp90 to interact with such a varied, yet specific set of molecules.

Hsp90 consists of 3 distinct regions-an N-terminal domain of approximately 25kDa (N-domain) that is connected to a 55KDa C-terminal domain via a highly charged 'linker' region (Figure 1.1). The N-terminal region is the best-studied, high-resolution crystal structures having been determined for this region in complex with ADP and with the inhibitors geldanamycin and radicicol. It was these studies that confirmed the presence of a site for ATP binding in this N-domain (Prodromou et al., 1997). This N-domain crystal structure showed Hsp90 to be a GHKC family ATPase, in common with the type II topoisomerases such as DNA gyrase B, the MutL DNA mismatch repair protein and the bacterial hisitidine kinase CheA (Prodromou et al., 1997).

Figure 1.1)

The structure of Hsp90

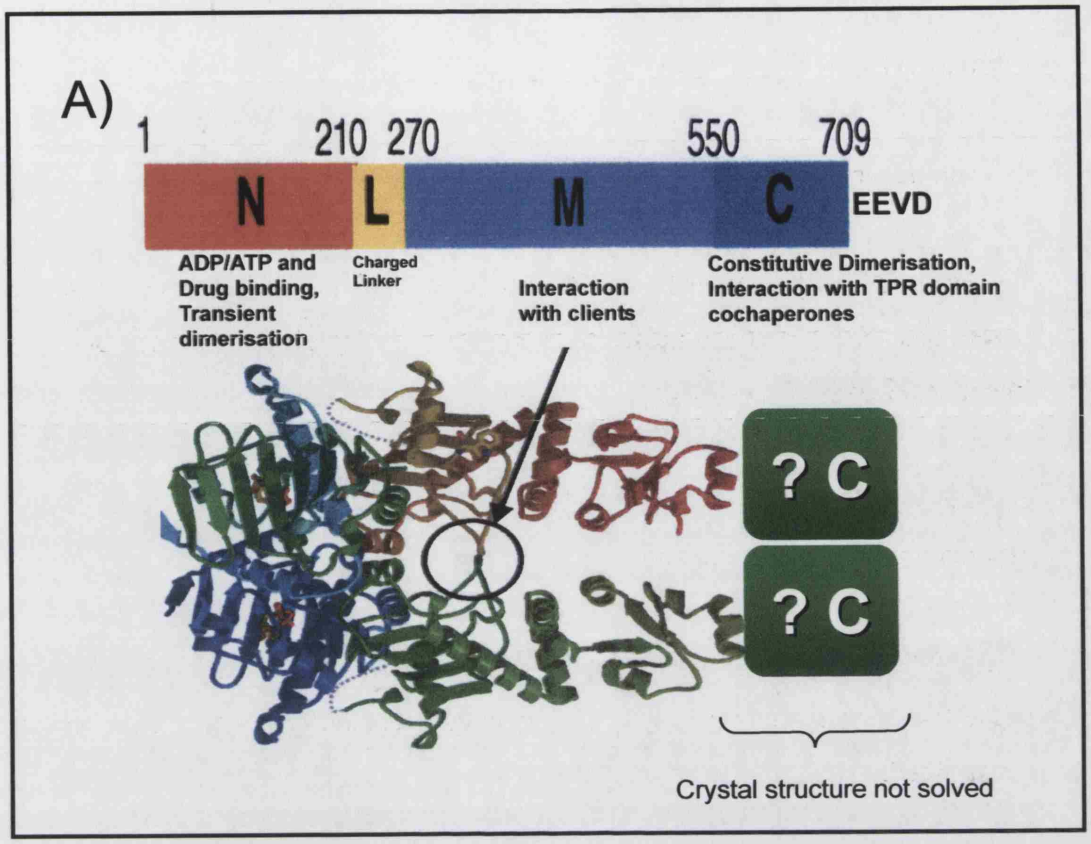
A) Schematic diagram of the Hsp90 structure. The N-terminal nucleotide binding domain (red) is linked to the middle segment (yellow) by a flexible and poorly conserved "charged linker" (blue).

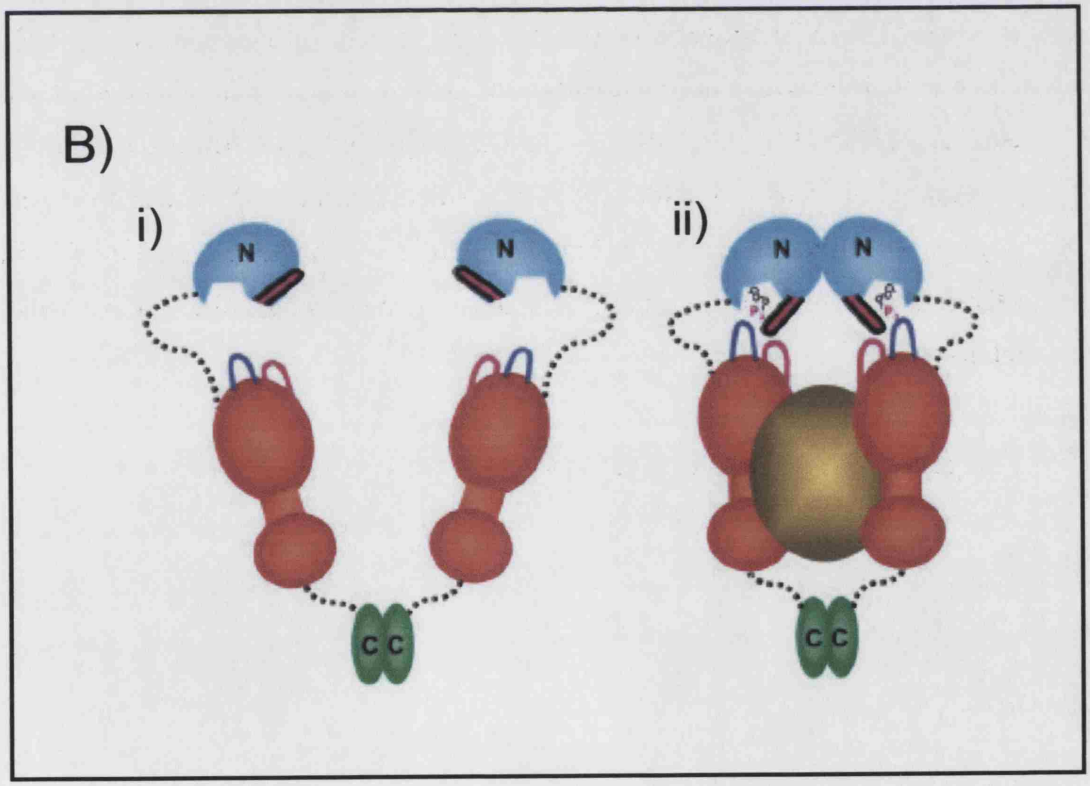
B) Model of the Hsp90 ATP-Dependent
Molecular Clamp Mechanism

(i) Hsp90 dimer in a "relaxed" state in the absence of ATP. The lid (black and magenta) in the N-terminal nucleotide binding domain is open, and the N-terminal domains are not constrained.

(ii) Binding of ATP causes lid closure, exposing a hydrophobic patch on the N-domain which associates with the equivalent in the other monomer. The exposed face of the lid itself is stabilized by interaction with a hydrophobic patch from the middle segment (magenta loop), which provides catalytic residues for the ATP-hydrolytic reaction (blue loop). The closed "tense" form interacts with client proteins (gold) to facilitate their activation and/or assembly with ligands, but how this occurs at the biochemical level is unknown.

(Reproduced from Meyer et al (2003))


A)
1
210
270
550
709
N
L
M
C
EEVD
ADP/ATP and Drug binding, Transient dimerisation
Charged Linker
Interaction with clients
Constitutive Dimerisation, Interaction with TPR domain cochaperones
? C
? C
Crystal structure not solved


B)
i)
N
N
C C
ii)
N
N
C C

Prior to this binding and hydrolysis of ATP had already been described as an essential part of the chaperone cycles catalysed by the Hsp70/DnaK and Hsp60/GroEL classes of chaperone. However, initial claims of ATPase and autophosphorylation activities of Hsp90 were largely discounted and explained as due to contamination from the trace amounts of kinases in Hsp90 preparations. Proof of the intrinsic ATPase of Hsp90 was not helped by the fact that pure Hsp90 has intrinsically low ATP turnover (for yeast Hsp82 Kcat=0.4-0.75min^{-1}) yielding a product (ADP) that binds the chaperone more tightly than the substrate (ATP) itself (Panaretou et al., 1998).

The issue was resolved by studies showing that the ATP/ADP binding to the N-domain of Hsp90 induces a structural change *in vitro* (Prodromou et al., 2000); also that disruption of ATP binding and ATPase activity, either by site-directed mutagenesis or through the use of inhibitors such as geldanamycin, causes complete loss of *in vivo* Hsp90 function (Panaretou et al., 1998; Prodromou et al., 1997; Prodromou et al., 1999).

1.2.1 The dimeric nature of Hsp90

Hsp90 functions as a dimer. Although the two protein molecules of this dimer are in constant association through their C-terminal regions, it is the binding of ATP that initiates the transient dimerisation of the N-terminal domains ((Prodromou et al., 2000), (Meyer et al., 2003); Figure 1.1). This conformational change is thought to 'trap' the client protein into a 'tight' complex. At the final stage of the chaperone cycle the ATP is hydrolysed, resulting in the opening of this molecular 'clamp' and the release of the now-activated client molecule (Figure 1.1b).

Direct evidence for the effect of ATP on Hsp90 conformation has come through biophysical measurements (Prodromou et al., 2000) of the conformational change produced by the addition of ATP. Without ATP, the molecules in the dimer only contact at their C-terminal end but with binding of ATP, both the N-terminal and C-terminal regions are in close contact (Maruya et al., 1999).

1.2.2 The Linker region of Hsp90

The charged 'linker' region, situated between the N-terminal and C-terminal domains, is the least conserved section of the Hsp90 protein. The linker in yeast Hsp90 consists of 4 or 5 repeats of the (E/D)(E/D(E/D)KK motif, but is dispensable for the essential function of the chaperone (Louvion et al., 1996).

1.2.3 The Mid Region (client binding) domain of Hsp90

A high-resolution structure of the middle domain of yeast Hsp82 was recently determined (Meyer et al., 2003), so that only the structure of the C-terminal constitutive dimerisation domain of the eukaryotic chaperone is at present unknown. This middle domain is probably the main binding region on Hsp90 for client proteins ((Young et al., 1997), (Meyer et al., 2003)). This viewpoint has been augmented by the knowledge that PKB/Akt and the glucocorticoid receptor bind to this region, along with the newly discovered co-chaperone Aha1p and its homologue Hch1p ((Lotz et al., 2003), (Panaretou et al., 2002)). Residues 435-525 have been suggested as the binding site of a second ATP molecule (Csermely et al., 1998), a site that becomes accessible to ATP when the N-terminal ATP-binding site is occupied with either geldanamycin or ATP analogues (Csermely et al., 1998). The current structural detail fails to prove the existence of this second nucleotide-binding site.

1.3 Hsp90 Co-chaperones

Activation of Hsp90 client proteins *in vitro* and *in vivo* is assisted by a number of co-chaperone proteins (Pratt and Toft, 2003). At least 10 have been identified in yeast; Sti1p, Sba1p, Cpr6p, Cpr7p, Cns1p, Ssa1p, Ydj1p, Hch1p, Aha1p and Cdc37p. The best-reconstituted client activation system is that of the steroid hormone receptor, where (in addition to Hsp90 itself) four co-chaperones are needed in order to attain a mature and activatable state (Hsp70, Hsp40, Hop and p23 (Pratt and Toft, 2003)). Each co-chaperone has different binding affinities for Hsp90, depending on the adenine-nucleotide determined conformation of the molecule and which client protein, if any, is bound at the time. Sti1p (p60/Hop in mammalian cells) acts as a 'scaffold' protein for Hsp90 and Hsp70 by means of its different tetratricopeptide (TPR) repeats (Young et al.,

1998). It also inhibits the inherent ATPase activity of the Hsp90 molecule, probably by keeping it in a nucleotide-free state (Prodromou et al., 1999). Sba1p, in contrast, will only bind Hsp90 when the latter is in ATP bound form and assists the final ATP dependent release of the activated client (Young and Hartl, 2000). Although loss of some co-chaperones in yeast generates no distinct phenotype, others are essential (Cns1p, Cdc37p) and defects in a third class of co-chaperones result in subtle changes to cell regulation.

1.4 The relationship between different Hsp90 isoforms

Hsp90 is evolutionarily extremely well conserved; a global alignment of all available Hsp90 sequences reveals a minimum amino acid identity of 40% (Gupta, 1995). In both *S.cerevisiae* and man, two isoforms of cytosolic Hsp90 exist (Hsp82 and Hsc82 in yeast, Hsp90α and Hsp90β in man). The two yeast proteins are 97% identical and probably as a result of the ancestral duplication of the *S.cerevisiae* genome (Wolfe and Shields, 1997) since many other yeasts have only one Hsp90. Hsp82 appears to be the equivalent of the human α isoform as both are induced strongly upon heat stress, the yeast Hsc82 isoform and the human β isoform being largely constitutive in nature. The α form readily dimerises, whereas the β form does so with much less efficiency (Grammatikakis et al., 2002). The human isoforms may have different functional roles in cell differentiation and development. A regulatory role of Hsp90α in muscle cell differentiation of zebrafish has been reported (Lele et al., 1999). Overexpression of Hsp90α inhibited cellular differentiation of embryonal carcinoma cells to trophoectoderm (Yamada et al., 2000). Hsp90β overexpression is observed throughout the germ cell lineage from very early stages of development to adult oocytes and spermatocytes (Hilscher, 1974). Later studies confirmed these observations in various models and different stages of development, indicating that Hsp90β is required for early embryonic development (Dugyala et al., 2002; Gruppi et al., 1991). The Hsp90α isoform, but not Hsp90β, is expressed extracellularly where it interacts with the matrix metalloproteinase 2 (MMP2). Inhibition of this extracellular Hsp90α decreases both MMP2 activity and invasiveness. This role for extracellular Hsp90α in MMP2 activation

indicates that cell-impermeant anti-Hsp90 drugs might decrease invasiveness without the concerns inherent in inhibiting intracellular Hsp90 (Eustace and Jay, 2004).

The two yeast Hsp90 isoforms, being 97% similar in sequence were thought to be equivalent in function until recently, when it was found that only overexpression of Hsp82, not the overexpression of the Hsc82 could rescue the temperature-sensitivity of a yeast that expresses a HSF lacking the CTA domain, (the *HSF (1-583)* mutant; (Morano et al., 1999)).

1.5 Client proteins of Hsp90

Hsp90 is needed for the activity of a wide variety of substrates or 'client proteins'. Examples are Helix-loop-helix transcription factors (the dioxin receptor); β,γ subunits of trimeric G-proteins; tumour suppressors (p53 mutant forms only, Rb); protein kinases (wee-1, casein kinase, Raf1, Cdk4, eIF2α kinase, DNA-PK), and several other key regulatory proteins such as Hepatitis B reverse transcriptase and telomerase (Pearl and Prodromou, 2000).

Two classes of client proteins remain the best well-studied, nuclear hormone receptors and protein kinases. Nuclear hormone receptors exist in an inactive, Hsp90-associated state in the cytosol where they are competent to bind ligand. It is thought their association with the Hsp90 chaperone complex induces the formation of the steroid binding cleft, since in its absence the receptor is not steroid responsive (Pratt and Toft, 2003). Upon ligand binding, Hsp90 releases the activated dimeric receptor which then translocates to the nucleus, where it binds to DNA and elicits steroid-responsive transcription. Well-studied Hsp90-dependent hormone receptors are those that bind glucocorticoid, oestrogen, progesterone, androgen; but not those binding retinoic acid, thyroid or vitamin D. Activation studies of the former group have shown that their ligand-binding clefts must be opened in two ATP dependent steps for the activation of the receptor (Morishima et al., 2000).

Another well-studied class of Hsp90 client proteins are the tyrosine kinases, examples being src, fyn, fgr, erbB; but not abl. In general Hsp90 appears to potentiate the capacity of clients to be activated in response to either the binding of another protein (e.g. cyclin D for the Cdk4 cyclin-dependent kinase; Ras-GTP for Raf-1), the binding of a ligand (e.g. steroid for steroid receptors); or the insertion of a cofactor (e.g. haem for endothelial nitric oxide synthase (eNOS)).

1.6 The role of Cdc37p in the binding of Hsp90 to kinases

Most Hsp90-protein kinase interactions require the function of the Cdc37p (p50) co-chaperone. The yeast *CDC37* gene was originally identified from a temperature-sensitive cell-division cycle mutant in which cells arrest in G1 at the non-permissive temperature (Breter et al., 1983). Cdc37p and Hsp90 bind to, and are essential for, the activity of many (though not all) protein kinases. This is one of the reasons that the inhibition of Hsp90 in the cell has such wide effects on such processes as cell cycle regulation, signal transduction, transcription and DNA/protein synthesis. It is not known what the criteria are for a protein kinase to bind, or be dependent on, Hsp90. Recent studies have suggested that it is the well-conserved N-terminus of Cdc37p that binds kinases (MacLean and Picard, 2003), but how certain kinases are chosen to be clients of Hsp90 while others are not has always been a mystery. (Zhao et al., 2004) have shown that Cdc37p binds to a Gly-X-X-Gly motif in target kinases.

The first yeast protein conclusively demonstrated to require Hsp90 for function was Ste11p (Louvion et al., 1998). Ste11p functions as a MAP kinase kinase kinase in the pheromone response MAP kinase signal transduction pathway. Some Hsp90 mutants are defective in pheromone signalling due to the lack of this Ste11p function (Louvion et al., 1998) and Ste11p can be immunoprecipitated with Hsp90 in cell extracts, although this interaction is weak reflecting the transient nature of the interaction. It has recently been shown that Ste11p requires the binding of a variety of co-chaperones such as Cpr7p, Sse1p and Ydj1p for full functionality (Lee et al., 2004).

1.7 MAP kinase involvement in stress regulation

MAP kinases are major components of pathways controlling such varied cellular responses as embryogenesis, cell differentiation, cell proliferation and cell death (Pearson et al., 2001). They are activated through phosphorylation cascades consisting of a three-kinase module, a MAP kinase kinase kinase (also known as a MEKK), a MAP kinase kinase (or MEK) and the MAP kinase itself. Provided each successive protein in the phosphorylation cascade is more abundant than its upstream regulator, signal amplification is achieved (Errede et al., 1995). A characteristic of MAP kinases is that they are activated by dual phosphorylation (thereby attaining their active form) by the appropriate MAP kinase kinase at a TXY motif (for example; TEY in ERK2 and TGY in the stress-activated p38). Most MAP kinases are phosphorylated on the tyrosine first then the threonine (Ferrell and Bhatt, 1997; Robbins and Cobb, 1992). Since the singly phosphorylated protein is not active and must accumulate before phosphorylation of the threonine residue can occur, a threshold is established, the kinases becoming rapidly converted to the active state, as the threonine becomes phosphorylated (Ferrell and Bhatt, 1997).

1.7.1 The activation of MAP kinases

The crystal structures of unphosphorylated and phosphorylated ERK2 reveal that MAP kinases undergo a conformational change upon phosphorylation (Canagarajah et al., 1997). The dually-phosphorylated lip flips up 9Å into the domain interface promoting domain closure (see Figure 1.2). This change brings the key catalytic residues into alignment, such that the ERK2 kinase becomes activated and partially functional (Canagarajah et al., 1997). This conformational change also induces ERK2 to homodimerise via a leucine rich region (Figure 1.2). Many MAP kinase substrates are dimers, and the active MAP kinase dimer is thought to first engage in a docking interaction with its various specific substrates and then phosphorylate these substrates at

Figure 1.2)

Activation mechanism of MAP kinases

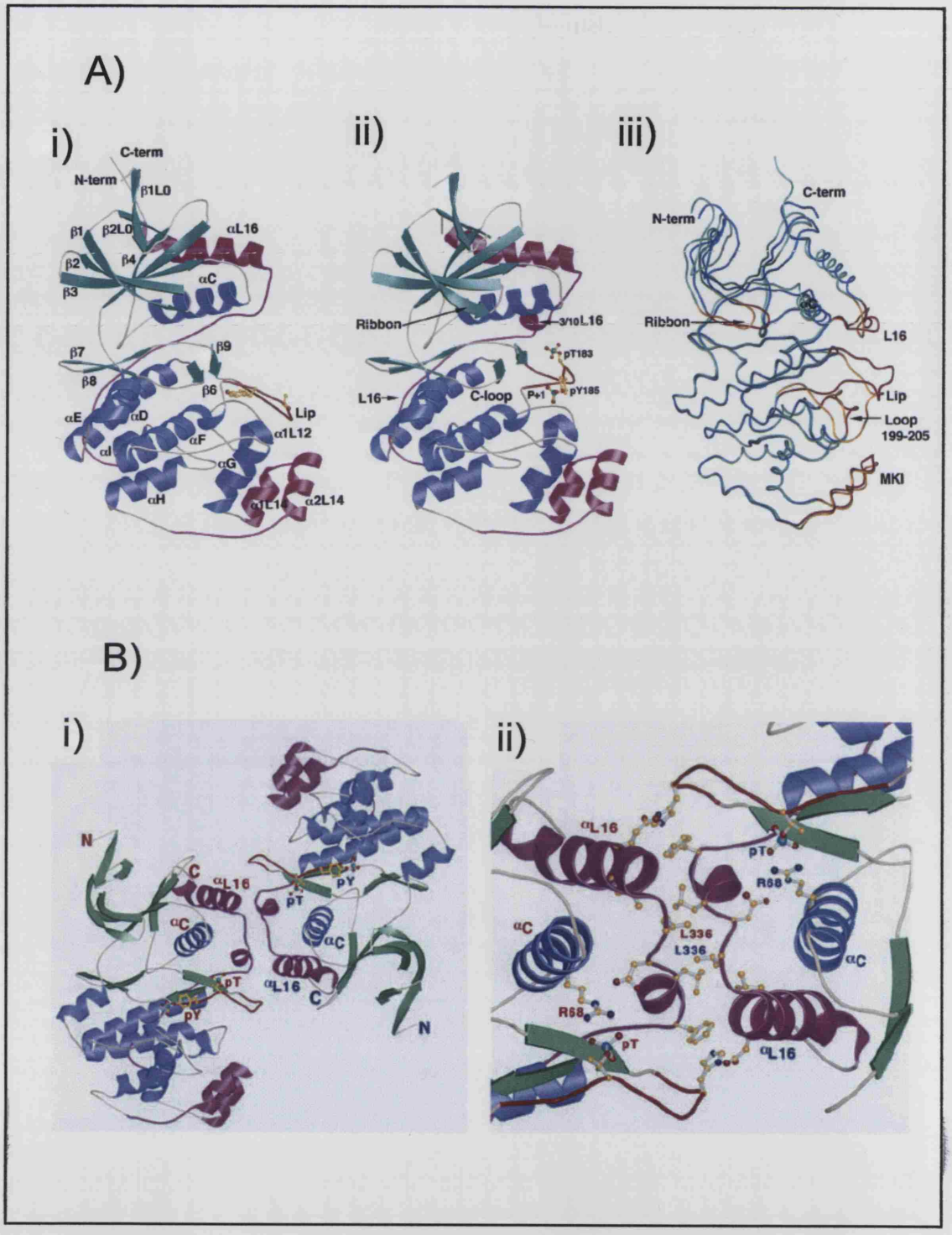
A) Low-Activity and Doubly Phosphorylated ERK2

Ribbon diagram of (i) ERK2 and (ii) ERK2-P2. The N-terminal domain (residues 1–109 and 320–358) is formed largely of *b* strands (green) and two helices, C (blue) and *a*L16 (magenta). The C-terminal domain (residues 110–319) is mostly helical (blue), contains the phosphorylation lip (red) and the MAP kinase insertion (magenta, labeled MKI), and is the locus of the P*I*1 site, the catalytic loop (residues Arg-147–152, labeled C loop). The side chains of Thr-183, Tyr-185, pTyr-183, and pTyr-185 are shown. (iii) Superposition of ERK2 (green and gold) and ERK2-P2 (blue and red) using corresponding C*a* atoms within the C-terminal domain (residues 109–171, 205–245, 272–310), drawn using SETOR (Evans, 1993). The molecules have been rotated by about 90*8* about the vertical axis relative (A) and (B) to show domain rotation and closure. Refolded segments are highlighted in red and gold, respectively. (Reproduced from Canagarajah 1997).

B) **ERK2 Dimers**

(i) The structure of the ERK2 dimer shows the active sites on the same face and the substrate binding groove distal to the dimer axis. The *b* strands are in green, *a* helices in blue, and L16 and the MAP kinase insert in magenta. Phosphorylated residues, T183 and Y185, are indicated by P-T and P-Y in yellow. (ii) Leucine residues 333, 336, and 344 in L16 contact each other in the dimer. H176 in the phosphorylation lip of one molecule and E343 in L16 of the other form an ion pair.

(Reproduced from Khokhlatchev 1998).


A)
i)
C-term
N-term
β1L0
β1
β2L0
αL16
β2
β4
αC
β3
β7
β9
β8
β6
Lip
αE
αD
α1L12
αI
αF
αG
αH
α1L14
α2L14
ii)
Ribbon
3/10L16
pT183
L16
C-loop
P+1
pY185
iii)
C-term
N-term
Ribbon
L16
Lip
Loop
199-205
MKI
B)
i)
N
C
αL16
pY
pT
αC
αC
pT
αL16
C
pY
N
ii)
αL16
pT
R58
αC
L336
L336
αC
R58
pT
αL16

sites quite distinct from the sites of docking ((Tanoue and Nishida, 2003), (Khokhlatchev et al., 1998)). An active MAP kinase dimer may have substrates both in the nucleus (where it binds and activates transcription factors) and the cytosol (where in the case of the yeast MAP kinase Slt2p it may cause cytoskeletal changes (van Drogen and Peter, 2002). It also engages in a docking interaction with inactivating phosphatases.

1.7.2 The yeast cell wall

S. cerevisiae uses a large quantity of its metabolic energy in building a cell wall, a structure that accounts for about 20-30% of the cell dry weight. This wall has an important skeletal function, determining cell morphology and, in the case of mating cells, cell-cell recognition. The cell wall structure consists of different components, one of the most important being a three-dimensional network of largely β-1,3-linked glucan chains (up to 40 mannoses in length) which surround the entire cell. It is this network that provides the mechanical strength of the wall (Kapteyn et al., 1999). The mature β1,3-glucan chains have β1-6 linked branches of 2-3 mannoses, the latter being frequently phosphorylated to give terminal phosphomannoses. The mature β1,6-glucan linked branches are found on the outside of the framework and interlink glycosylphospatidylinositol (GPI)-cell wall proteins with the framework (Smits et al., 1999). In the region of the bud scar this skeletal framework is reinforced by chitin chains, which are found close to the plasma membrane.

1.7.3 The cell integrity (Slt2p MAP kinase) pathway

The cell integrity pathway is activated during changes in cellular morphology, controlling the expression of genes of cell wall biosynthesis (Hohmann, 2002). It is not a single straight cascade, but rather a network of interacting signalling routes that converge on Pkc1p, the start of a classic MAP kinase signalling module. Mutants with defects in this pathway display, with differing degrees of severity, cell wall defects. This manifests itself as the cells being swollen, sensitive to calcofluor white and caffeine, and unable to grow at high temperatures, defects that can be counteracted by osmotic stabilisation. Such mutants lyse when exposed to pheromone (Errede et al.,

1995), or caffeine and calcofluor white (agents which seem to specifically act on the cell wall). The pathway is activated with different timing and kinetics, by hypo-osmotic shock (Davenport et al., 1995), by heat stress (Kamada et al., 1995), during bud emergence, upon exposure to mating pheromone (Errede et al., 1995)and by treatments that lead to cell wall perturbation (e.g. lyticase and calcofluor white) (de Nobel et al., 2000a).

1.7.4 The cell surface receptors

When the cell wall is weakened, a signal must be transduced from the cell surface to the nucleus in order to reprogramme gene expression, so that reinforcements can be made to the cell wall to prevent cell lysis (de Nobel et al., 2000b). Candidates for plasma membrane transducers of a weak cell wall signal include Wsc1p (Slg1p, Hcs77p) (Delley and Hall, 1999; Gray et al., 1997; Jacoby et al., 1998), Wsc2p-4p (Zu et al., 2001), Mid2p (Ketela et al., 1999; Philip and Levin, 2001) and Mtl1p (Rajavel et al., 1999). These proteins (all type 1 transmembrane proteins, with a single transmembrane domain) have a serine/threonine rich intracellular domain and an extracellular domain that is heavily glycosylated. Although structurally very similar, they can be divided into two groups based on sequence similarity; Wsc1p-4p and Mtl1p, Mid2p. The main difference is that Mid2p and Mtl1p lack a cysteine-rich region found between the signal sequence and the serine/threonine-rich domain in Wsc proteins. There is evidence that these two groups of sensors have overlapping, but not identical functions. For example, Mid2p is required for the phosphorylation of Slt2p MAP kinase under heat stress, whereas Wsc1p is not (Martin et al., 2000). It has been speculated that Wsc1p-Rom2p-Rho1p and Pkc1p stimulates a rapid redistribution of the actin cytoskeleton and the β-1, 3 glucan synthase so as to repair cell wall damage over the entire cell surface, whereas the Slt2p MAP kinase cascade is required for repair of localised damage and the polarised cell wall growth found in budding and schmoo formation (Hohmann, 2002). The Rho1p GTPase is also a regulatory subunit of glucan synthase (Figure 1.3).

1.7.5 Protein Kinase C (Pkc1p) in yeast

The first component of the cell integrity pathway to be isolated was *PKC1*, the only yeast homologue of the mammalian protein kinase C isoforms (Levin et al., 1990). This *PKC1* gene seems to be a more generalised version than its mammalian counterparts, since it contains most of the domains found in the different mammalian protein kinase C isoforms. The phenotype of cells lacking *PKC1* function is a uniform growth arrest with small buds, indicating an early S-Phase cell cycle arrest. These cells will though grow on medium containing sorbitol at 30°C or lower, suggesting a morphological checkpoint to monitor turgor pressure in the cell acting at START.

1.7.6 The cell integrity MAP kinase module

Genetic analyses also revealed components operating downstream of Pkc1p (Gustin 1998). A constitutively active form of a MAP kinase kinase kinase was isolated that suppressed the growth defect of a *pkc1*Δ mutant ((Lee and Levin, 1992)). Loss of this kinase (named Bck1p for bypass of C kinase), results in temperature sensitivity, but less severe temperature sensitivity than that of a *pkc1*Δ mutant. The genes for the downstream components of this cascade (*MKK1*, *MKK2* and *SLT2*) were isolated as multicopy suppressors of the *pkc1*Δ mutation. Mkk1p and Mkk2p are functionally redundant and are classic MAP kinase kinases with 59% sequence identity to each other. *SLT2 (MPK1)*, a gene encoding a 484 amino acid MAP kinase (M_r55636), was originally isolated by complementation of the lysis sensitive *lyt2* mutant (Torres et al., 1991). Epistasis analysis confirmed the signalling sequence of the pathway (Figure 1.3).

Yeast two-hybrid analysis suggests that the MAP kinase kinases Mkk1p and Mkk2p are important in mediating the complex formation between the pathway components, very much like Pbs2p of the *HOG*1 pathway. A genuine scaffold protein for the *PKC1* pathway (Spa2p) has only recently been isolated (van Drogen and Peter, 2002).

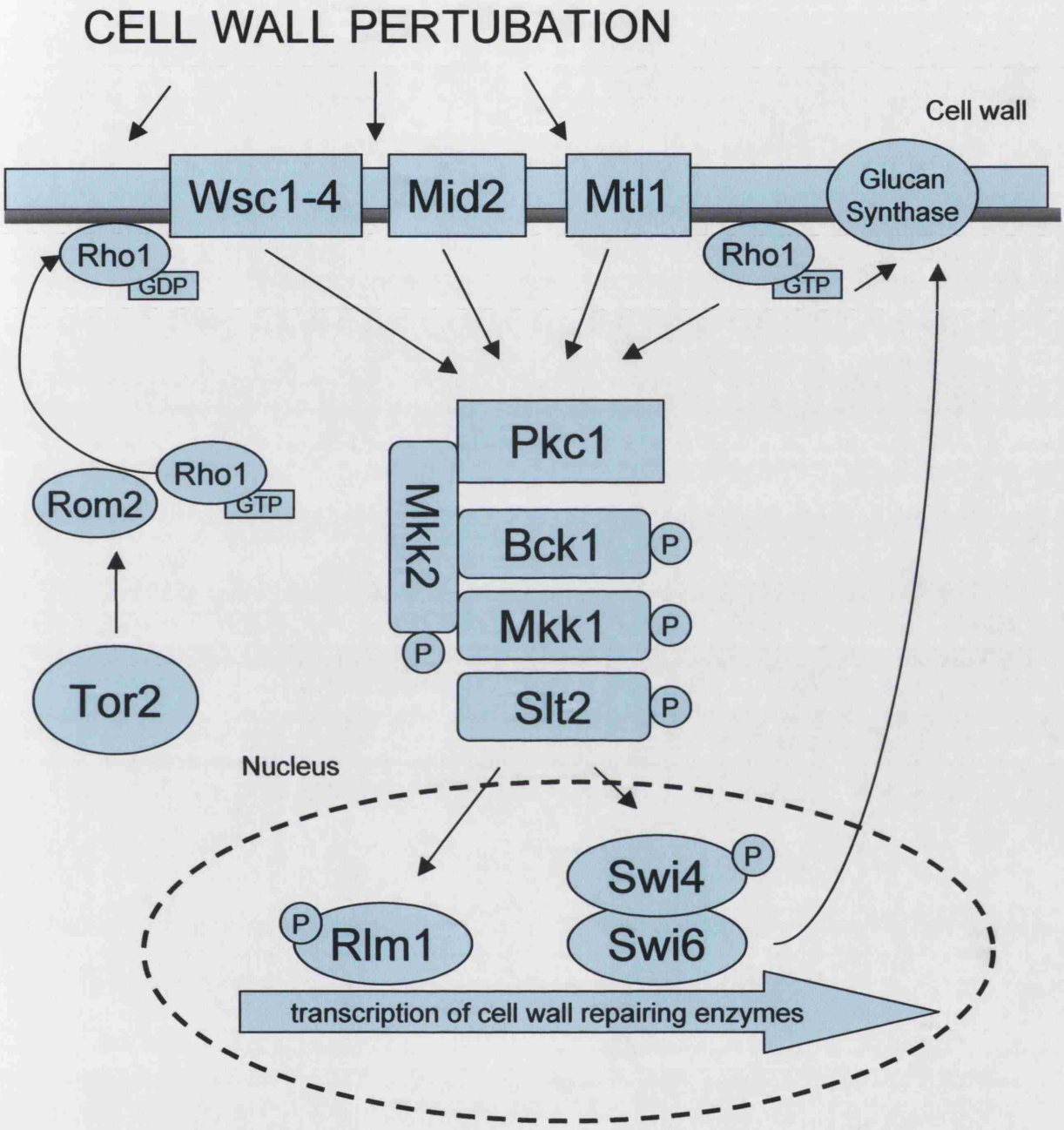


Figure 1.3)

The cell integrity (Pkc1p) pathway in *S. cerevisiae*. Cell stress sensors transmit their signals to the MAP kinase cascade resulting in its consecutive phosphorylation. The terminal MAP kinase, Slt2p becomes phosphorylated, and in the nucleus activates downstream targets such as Rlm1p and the SBF complex (consisting of Swi4/6). This leads the induction of cell wall repairing enzymes, resulting in a reinforced cell wall.

1.7.7 The Slt2p MAP kinase

Loss of Slt2p leads to a thermo-sensitive autolytic lethal phenotype that can be prevented by osmotic stabilisation with 1M sorbitol (Martin et al., 1993). Slt2p is a serine-threonine protein kinase that carries all the characteristics of MAP kinase enzymes. A Gly-X-Gly-X-X-Gly motif is present in the N-terminus of the protein, followed by a conserved ATP binding lysine residue (K54 in Slt2p). Mutation of this lysine to an arginine (K54R) creates a catalytically dead version of Slt2p that will not suppress the temperature-sensitive phenotype of the *slt2Δ* mutant (Torres et al., 1991). Slt2p is dually phosphorylated by Mkk1/2p at the threonine and tyrosine of its TEY phosphorylation motif in response to activation of the *PKC1* pathway. Lee et al (Lee et al., 1993) demonstrated that the threonine in this motif is absolutely required for Slt2p function (The T190A mutant displays a *slt2Δ* phenotype); yet interestingly, it appears that the tyrosine (at position 192) is not essential, although the cells are slightly osmoremedial. Work on the other stress-activated MAP kinase of yeast, Hog1p, seems to show a similar phenomenon (Bell and Engelberg, 2003). Slt2p shares high similarity with the mammalian MAP kinase ERK2 (50% identity over the MAP kinase domain region), but an even greater similarity with the other four yeast MAP kinases Hog1p, Fus3p, Kss1p and Smk1p. What sets Slt2p apart from the other MAP kinases of yeast is its extraordinarily long C-terminal tail (155 amino acids compared with 29 in Fus3p). This C terminal domain follows the MAP kinase module and contains a polyglutamine tract consisting of some 16 glutamines. The function of this polyglutamine tract is unknown, although it may target Slt2p in the cell. This tail section can be removed without appreciable loss of Slt2p function ((Soler et al., 1995), work done in this thesis), its possible function is discussed further in Chapter 4.

1.7.8 Slt2p and the cytoskeleton

The first link between the Pkc1p pathway and the cytoskeleton was found when Rho1p was detected at the cortical actin patches in the growing daughter cell (Yamochi et al., 1994). It was later shown that the Mkk1p and Mkk2p, along with Slt2p, reside in a multiprotein complex at these actin patches (van Drogen and Peter, 2002). These

complexes also contain Spa2p, Pea2p and Bni1p. Spa2p acts as a scaffold-like protein to recruit the Slt2p MAP kinase module to these sites of polarised growth (van Drogen and Peter, 2002). In this way there is an integration of processes encompassing responses to cell wall damage, mating, budding and pseudohyphal development. *slt2*Δ mutants have defects in actin organisation and accumulate secretory vesicles at 37°C (Mazzoni et al., 1993). Cells deleted for both Sac1p (an integral membrane protein with an important role in the ER) and Slt2p arrest as large-budded cells because they fail to separate at the end of mitosis. This appears to be caused by an increased deposition of chitin at the septum area and correlates with a mislocalised accumulation of the Chs2p chitin synthase at the cell periphery (Schorr et al., 2001; Tahirovic et al., 2003).

1.7.9 Slt2p and Cell Cycle Control

Cdc28p, the cyclin-dependent kinase that regulates the yeast cell cycle, is known to activate the Pkc1p pathway, leading to activation of Slt2p (Marini et al 1996). In this way Cdc28p mediates an enhanced expression of cell wall genes in the G1 phase of the cell cycle via the Swi4/Swi6p (SBF) transcription factor (itself also a target of Slt2p, Figure 1.3). Slt2p also controls Cdc28p by inhibitory phosphorylation of the tyrosine phosphatase Mih1p (Harrison et al., 2001). Mih1p action counteracts the effects of the inhibitory phosphorylation of the mitotic Cdc28p/cyclin B complex by the *S.cerevisiae* Wee1-like kinase, Swe1p. This phosphorylation is maintained with operation of the of the morphogenesis checkpoint (activated by actin depolymerising agents such as latriculin B). Therefore, it appears that Slt2p controls both Cdc28p activity, via Mih1p, and cell wall remodelling during cell growth at the transcriptional level via the SBF complex.

1.7.10 ***Transcription factor targets of the cell wall integrity pathway***

The cell integrity pathway directs activation of at least two transcription factors, Rlm1p and SBF. Rlm1p is a member of the MADS (Mcm1-Agamous-Deficiens-serum response factor) box proteins and is the main transcriptional regulator of cell wall genes (Jung et al., 2002). This 676 amino acid protein has an N-terminal end containing the DNA binding MADS box, a mid section being required for Slt2p dependent phosphorylation and a C-terminal part containing the transcriptional activation domain (Watanabe et al., 1997). It was originally identified from a mutation which supressed the toxicity caused by the overexpression of the hyperactive (S386P) allele of Mkk1p (Watanabe et al., 1995). An Rlm1p fusion mediating Slt2p-independent transcriptional activation of Rlm1p-dependent genes (an AD-Rlm1p fusion which carries the transcriptional activation domain of Gal4p (AD)), suppresses the temperature and caffeine sensitive phenotypes of *bck1Δ* and *slt2Δ* mutants (Watanabe et al., 1995). Recent data by Jung et al 2002, shows that Slt2p binds Rlm1p (using the conserved MAP kinase docking site (LRVRIP) that Rlm1p shares with MEF2 isoforms) so as to catalyse the phosphorylation of Rlm1p on residues Ser427 and Thr439. By monitoring the expression of an Rlm1p-dependent-LacZ reporter construct, it can be shown that Rlm1p activity increases 2 to 3-fold under heat-stress, while Slt2p activity increases up to 100-fold (Watanabe et al., 1997). This implies that Rlm1p provides a strong, constant induction of cell wall repairing genes in dividing yeast cells.

The other known transcriptional target of Slt2p, SBF, consists of a DNA-binding subunit (Swi4p) and a regulatory subunit (Swi6p) (Primig et al., 1992). SBF is activated by Cdc28p/Cln1p late in G1 and stimulates the expression of important cyclin genes such as *CLN1*, *CLN2*, *PCL1*, and *PCL2*, along with cell wall genes that are also Rlm1p-activated (Igual et al., 1996). The *swi4Δ* mutant displays phenotypes characteristic of a weak cell wall, defects that can be supressed by overexpression of Pkc1p (Igual et al., 1996).

Interestingly, Slt2p is not the only potential Rlm1p interactor. A two-hybrid screen isolated Mlp1p as an Rlm1p interactor. Mlp1p displays 53% sequence similarity to

Slt2p, its closest homologue, and also binds to the same region of Rlm1p as Slt2p. Deletion of Mlp1 appears to have no effect, but Mlp1p overexpression appears to decrease the caffeine sensitivity of a *bck1*Δ mutant, suggesting that Slt2p and Mlp1p might have overlapping functions (Watanabe et al., 1997). Since lysine replaces threonine in the consensus TEY phosphorylation site of the latter, Mlp1p is not classified as a MAP kinase. Also the key catalytic lysine (K54 in Slt2p) is replaced with an arginine, suggesting that Mlp1p may not be an active protein kinase.

1.7.11 Genes induced for cell wall biosynthesis

Activation of the Pkc1p pathway also results in the induction of Fks1p and Fks2p, the catalytic subunits of β-1, 3 glucan synthase (de Nobel et al., 2000b). Mutants in which either one of these glucan synthase genes are deleted are still viable, but the removal of both genes results in cell death. Thus in the absence of Fks1p, Fks2p becomes essential, and vice versa. Fks1p expression is dependent on the Ca^{++}-regulated phosphatase calcineurin (Mazur et al., 1995) and is mediated via the transcription factor Crz1p-Tcn1p. After either pheromone or heat treatment, the intracellular calcium levels are increased along with activity of the Pkc1p pathway (apparent from increased levels of phosphorylated Slt2p). Thus increased calcium signalling and increased activity of the Pkc1p pathway frequently appear to be co-ordinated.

1.8 Big-Mitogen activated protein kinase1 (BMK1/ERK5)

So conserved are the MAP kinases that it is possible that the functions of the yeast enzymes might be replaced by certain of their mammalian counterparts. 'Big MAP kinase' (also known as BMK1 or ERK5) may be the functional counterpart of the yeast Slt2p (a possibility investigated as part of this study; Chapter 5). BMK1 was originally identified by two groups. Zhou et al used a yeast two-hybrid screen using MEK5 as the bait, whereas Lee et al used a degenerate PCR strategy (Lee et al., 1995; Zhou et al., 1995). Like other MAP kinases, BMK1 has a conserved kinase domain containing the classic MAP kinase TEY activation sequence, but as with Slt2p of yeast, BMK1 is set apart from other mammalian MAP kinases by its unusually large size (816 amino acids). As mentioned previously, although the N-terminal catalytic kinase domain of around

319 amino acids is conserved throughout MAP kinases and indeed eukaryotic species, the C-terminal appears to depend on the function of the enzyme.

The C-terminal tail of BMK1 is very large (around 400 amino acids) as compared to 28 amino acids in ERK2, and displays no similarity to other known protein sequences. In addition, it contains 10 consensus sites for MAP kinase phosphorylation and two proline rich regions. The second proline rich region (124 amino acids with 44 prolines) contains several proline-alanine repeats, a motif which is present in myosin light chain kinase. It is this repeat which has been shown to interact directly with actin, targeting the kinase to specific locations in the cell (Yan et al., 2001). Recently it has been shown that this C-terminal tail is responsible for both localisation and transcriptional activation potential (Kasler et al., 2000; Yan et al., 2001).

The MAP kinase module of BMK1 has high similarity to that of yeast Fus3p and Kss1p, also the human ERK's 1 and 2. Loop-12 (between kinase domain 7 and 8) is believed to be important for kinase regulation, and is often used to compare MAP kinases to each other. Based on this loop-12 region, yeast Slt2p is the protein most homologous to BMK1 (Lee et al., 1995). BMK1 is widely expressed and activated in response to a variety of signals including chemical activators such as serum, EGF, nerve growth factor, lysophosphatidic acid and phorbal ester; oxidative stress such as hydrogen peroxide and UV radiation; and physical stress such as the osmotic stabiliser sorbitol, vascular shear stress, and ischemia (Abe et al., 1996; Yan et al., 1999). Studies have shown that the dual phosphorylation of BMK1 leads to activation of a number of substrate proteins, including MADS box transcription factors, such as MEF2, myocyte enhancer 2A and C, and the ETS-like transcription factor SAP1a. The phenotype of BMK1-null mice strongly suggests that BMK1 is important for blood vessel maintenance. Specifically, BMK1-null mice display defective endothelial cell (EC) morphology, blood vessel formation and cardiac development leading to embryonic lethality (Hayashi et al., 2004). Indeed, it has been shown recently that BMK1 activation prevents EC apoptosis by stimulating the phosphorylation of Bad in the cytoplasm and inhibiting capsase-3 activity (Pi et al., 2004).

1.9 Outline of this research project

Hsp90 remains one of the most interesting molecular chaperones. During the course of this research, the crystal structure of the mid-section of Hsp90 was determined (the region thought to bind client proteins (Meyer et al., 2003)). Despite this, it is still unclear what determines Hsp90-dependence in client proteins. The aim of this project was to identify a model client protein of Hsp90 that might be used to investigate this Hsp90 dependence. The phenotypic effects in yeast of loss of the HSF CTA domain (Chapter3); also single point mutations in Hsp82 expressed as the sole Hsp90 of the cell, mutations that partially compromise Hsp90 function (Chapter 4) revealed Slt2p MAP kinase to be such a model client. This was the first identification of Hsp90 dependence in a kinase of the MAP kinase class (although a MAP kinase-related protein, mammalian MOK has been shown to bind Hsp90). The nature of this model Hsp90-client protein interaction was studied using techniques such as yeast two-hybrid and immunoprecipitation (Chapter 4). It is also shown that BMK1 can partially provide Slt2p MAP kinase function in yeast (Chapter 5).

2 Materials and methods

2.1 Growth media and culture conditions

Yeast cultures were either grown in rich media (YPD) or synthetic defined minimal media (SD). The recipes for all yeast media are given below (all % values are w/v):

YPD: 2% D-glucose, 2% Bacto-peptone, 1% Bacto-yeast extract.

SD: 2% D-glucose, 0.67% yeast nitrogen base (without amino acids) plus one or more of the following auxotrophic amino acids where required; adenine (20mg/l), L-histidine (20mg/l), L-leucine (30mg/l), L-lysine (30mg/l), L-tryptophan (20mg/l), uracil (20mg/l).

Dropout media: 26.7g of SD base (Clontech) and appropriate amount of complete synthetic media supplement lacking the required amino acids were added per litre of required media.

Plates were left on the bench for 1-2 days after pouring to dry. Liquid media cultures were grown at the appropriate temperature with rapid agitation in a media/volume ratio of 1/5 of the flask volume. Yeast strains were maintained in frozen stocks in 2X YPD + 15% glycerol at –70°C. All solutions and glassware were sterilised by autoclaving at 15psi for 20min.

Table 2.1. The yeast strains used in this study

Strain	Genotype	Reference
Strains used to investigate HSF(1-583) allele		
W303-1a	MAT**a** *ura1-1 trp1-1 leu2-3,112 his3-11 ade2-1 can1-100 ssd1-3*	(Watanabe et al., 1997)
NSY-A	W303-1a *hsf1::LEU2* (pRS314-*HSF*)	(Morano et al., 1999)
NSY-B	W303-1a *hsf1::LEU2* (pRS314-*HSF*(1-583))	(Morano et al., 1999)
dHSF3(HSF)	MAT**α** *ura3-0 his3-Δ1 leu2-Δ0 lys2-Δ0 trp1-ΔkanMX4 hsf1-ΔkanMX4* (pRS314-*HSF*) [b]	This study
dHSF3(HSF1-583)	MAT**α** *ura3-0 his3-Δ1 leu2-Δ0 lys2-Δ0 trp1-ΔkanMX4 hsf1-ΔkanMX4* (pRS314-*HSF*(1-583) [b]	This study
Strains with altered Hsp90 function		
P82a	W303-1a *hsc82::LEU2 hsp82::LEU2 HIS3-GPD-HSP82*[a]	(Nathan and Lindquist, 1995)
*hsp82*T22I	W303-1a *hsc82::LEU2 hsp82::LEU2 HIS3-GPD- hsp82(T22I)*[a]	(Nathan and Lindquist, 1995)
*hsp82*A41V	W303-1a *hsc82::LEU2 hsp82::LEU2 HIS3-GPD- hsp82(A41V)*[a]	(Nathan and Lindquist, 1995)
*hsp82*G81S	W303-1a *hsc82::LEU2 hsp82::LEU2 HIS3-GPD- hsp82(G81S)*[a]	(Nathan and Lindquist, 1995)
*hsp82*T101I	W303-1a *hsc82::LEU2 hsp82::LEU2 HIS3-GPD- hsp82(T101I)*[a]	(Nathan and Lindquist, 1995)
*hsp82*G170D	W303-1a *hsc82::LEU2 hsp82::LEU2 HIS3-GPD- hsp82(G170D)*[a]	(Nathan and Lindquist, 1995)
*hsp82*G313S	W303-1a *hsc82::LEU2 hsp82::LEU2 HIS3-GPD- hsp82(G313S)*[a]	(Nathan and Lindquist, 1995)
*hsp82*E381K	W303-1a *hsc82::LEU2 hsp82::LEU2 HIS3-GPD-hsp82(E381K)*[a]	(Nathan and Lindquist, 1995)
*hsp82*A587T	W303-1a *hsc82::LEU2 hsp82::LEU2 HIS3-*	(Nathan and

	GPD- hsp82(A587T)	Lindquist, 1995)
P82a *slt2Δ*	P82a *slt2ΔkanMX4*	Thus study
T22I *slt2Δ*	*hsp82*T22I *slt2ΔkanMX4*	This study
PP30(pHSC82)	MAT**a** *trp1-289, leu2-3,112, his3-200,ura3-52, ade2-101^{oc}, lys2-801^{am}, hsc82::KANMX4, hsp82::KANMX4* [pHSC82]	(Piper et al., 2003a)
PP30(pHSP82)	MAT**a** *trp1-289, leu2-3,112, his3-200,ura3-52, ade2-101^{oc}, lys2-801^{am}, hsc82::KANMX4, hsp82::KANMX4* [pHSP82]	(Piper et al., 2003a)
Two hybrid strains		
PJ694-a	MAT**a** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	(James et al., 1996)
PJ694-α	MAT**α** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	(James et al., 1996)

2.2 Genetic techniques

2.2.1 Yeast transformation

Transformation of *S. cerevisiae* strains with DNA was performed chemically as described by (Gietz et al., 1995).

2.3 Molecular techniques

2.3.1 Restriction enzyme digest

0.1 to 4 μg DNA (in dH_2O or TE buffer) was digested with restriction endonuclease (1 to 5U/μg DNA) and with 1/10 volume 10x restriction buffer for 1 hour at specific temperature recommended by NEB.

2.3.2 Polymerase chain reaction (PCR)

The PCR was used for DNA amplification and site-directed mutagenesis. A 50 μl preparative PCR reaction contained 5 μl of 10X Expand™ high fidelity (HF) buffer with 15 mM $MgCl_2$ {20 mM Tris-HCL, pH 7.5, 100 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.5% Tween® 20(v/v), 0.5% Nonident® p40 (v/v), 50% glycerol (v/v)}, 100 μM dNTP mix (100 μM of each of dATP, dCTP, dTTP, dGTP (PE Applied Biosystems)), 100 pmol of each primer, and 1.14 unit Expand™ HF polymerase (Roche, Germany).

PCR conditions (Roche, Germany).

Number of cycles	**Conditions**
1x	Denature template 2 min. at 94°C
35-40x	Denaturation at 94°C for 30 sec Annealing usually at 45-65°C* for 30 sec Elongation at 72 or 68°C** for 45sec-8min Table 2.6.
1x	Final elongation at 72°C for min

* Annealing temperature depends on the melting temperature of the primers used.

** Elongation temperature depends on the length of amplification product: 72°C are used for amplification up to 3.0 kb; 68°C are used for amplification >3.0 kb.

PCR elongation time.

Elongation time	45 sec	1 min	2 min	4 min	8 min
PCR fragment length (kb)	<0.75	1.5	3.0	6.0	10.0

2.3.3 Dephosphorylation of 5' end of DNA and oligonucleotides

An appropriate amount of DNA or oligonucleotide was dephosphorylated using the Calf Intestine Alkaline Phosphatase (CIAP) kit (Promega).

2.3.4 Isolation and purification of DNA fragments from agarose gels

DNA fragments of 70 bp to 10 kb were extracted and purified from agarose gel using the QIAquick™ gel extraction kit (QIAGEN Ltd.).

2.3.5 Ligation of DNA fragments to plasmid vectors

Ligations were routinely carried out as described in (Maniatis et al., 1989).

2.4 Handling bacteria

2.4.1 E. coli growth media and culture conditions

E.coli strains were grown in LB (Luria-Bertani), (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl and 1mM NaOH final concentration) at 37°C. Cultures for plasmid transformation and selection were grown in LB plus ampicillin to a final concentration of 100μg/ml.

2.4.2 Preparation of competent E. coli

Competent *E. coli* cells were prepared according to a calcium chloride technique as described in (Maniatis et al., 1989). The competent cells were resuspended in ice cold 0.1M $CaCl_2$ plus 15% glycerol and stored in 0.1 ml aliquots at -70°C.

2.4.3 E. coli transformation

Competent *E. coli* cells were thawed on ice and 50 μl pipetted into a chilled 1.5ml eppendorf tube. An appropriate amount of plasmid DNA was added and the cells were incubated on ice for 30 minutes. The cells were heat-shocked at 42°C for 90 seconds and placed on ice for 3 minutes. The cell were then resuspended in 500 μl of SOC medium

(2% w/v tryptone, 0.5% w/v yeast extract, 10 mM NaCl, 2.5mM KCl, 10 mM $MgCl_2$, 20 mM $MgSO_4$, 20 mM D-glucose) and incubated at 37°C for 1 hour. The cells were centrifuged at 6,000g for 30 seconds. The pellet was resuspended in 100 μl of the supernatant and then plated on LB plus ampicillin to a final concentration of 100μg/ml and incubated at 37°C overnight.

2.4.4 High efficiency transformation by electroporation

Electro-competent E. coli cells were prepared as described in (Zabarovsky and Winberg, 1990).50 μl aliquots of electro-competent E. coli cells and an appropriate amount of DNA (10 μl) were added to a Flowgen cuvette and electroporated (at 2500 V, 0.25 mF, 201 Ω, 5 msec) in an EasyjectT+ electroporator (Flowgen, Inc.). Immediately after electroporation 1 ml of LB or SOC was added to the cuvette and quickly but gently resuspended the cells. The cells suspension was then incubated at 37°C for 45 minutes. The cells were centrifuged at 6000g for 30 seconds. The pellet was resuspended in 100 μl of the supernatant and then plated on LB plus ampicillin to a final concentration of 100μg/ml and incubated at 37°C overnight.

2.4.5 Preparation of plasmid DNA from E.coli.

Approximately 20-100 μg of high-copy plasmid DNA was obtained from 5ml or 3ml overnight cultures of *E.coli* using QIAprep® Miniprep or Midiprep kits respectively as described in the appropriate QIAprep® handbooks (QIAGEN Ltd.).

2.5 Procedures for nucleic acid analysis

2.5.1 Isolation of yeast total genomic DNA

Yeast genomic DNA was prepared as previously described by (Adams et al., 1997), except that proteinase K (200 μg/ml final concentration) was added at the point of spheroplast lysis. The lysate was extracted by phenol:chloroform:isoamylalcohol (25:24:1) and DNA was precipitated by addition of an equal volume of isopropanol. High molecular weight DNA was recovered by spooling and washed twice in 70% ethanol. DNA was resuspended in (TE or dH2O) and treated with ribonuclease A and

respooled before final resuspension in (TE or dH2O).

2.5.2 Quantification of DNA

DNA samples were quantified as described in (Maniatis et al., 1989). An A260 of 1.0 was taken to indicate 50μg/ml double stranded DNA, 37 μg/ml single stranded DNA. The ratio of A at 260 and 280nm was used to indicate nucleic acid purity.

Table 2.2. Sources of vectors used for this study

Name of Construct	Description	Source/Reference
YIL117c promoter-LacZ	Rlm1 activity-LacZ reporter	U.S. Jung (Jung et al., 2002)
pADc	Empty AD vector	S. Millson (Millson et al., 2003)
pBDc	Empty BD vector	S. Millson (Millson et al., 2003)
pYES2-E33A	Expression vector for E33A*hsp82*	B. Panaretou (Panaretou et al., 1998)
pYES2-D79N	Expression vector for D79N*hsp82*	B. Panaretou (Panaretou et al., 1998)
PG-1	Yeast expression vector	D.Picard (Louvion et al., 1996)
Hsp82(E33A)-6xHis-PG1	Expression vector for Hsp82(E33A)-6xHis	S. Millson (This study)
Hsp82(D79N)-6xHis-PG1	Expression vector for Hsp82(D79N)-6xHis	S. Millson (This study)
p2HG	Expression vector for Hsp82	D.Picard (Louvion et al., 1996)
ERK2/PGEX-6XT	Bacterial expression vector for Erk2p	C. Prodromou
BMK1-pOTB7	Vector containing BMK1 cDNA	IMAGE clone
pNV7W-MKK1^{P386}	Expression vector for constitutively-active allele of MKK1	K.Matsumoto (Watanabe et al., 1995)
pUG36	Yeast expression vector for *MET25* induced expression of GFP fusion proteins	D.Hegemann.
pUG34	Yeast expression vector for *MET25* induced expression of GFP fusion proteins	D.Hegemann

pUT36	Same as Pug36, but vector has been digested with Xba1 and religated to remove GFP section.	S.Millson (This study)
pUT34	Same as Pug34, but vector has been digested with Xba1 and religated to remove GFP section.	S.Millson (This study)

2.6 *Vectors created for this study*

2.6.1 *Constitutively active Rlm1 expression vector (AD-Rlm1p) expression vector, AD-Rlm1-pUT36*

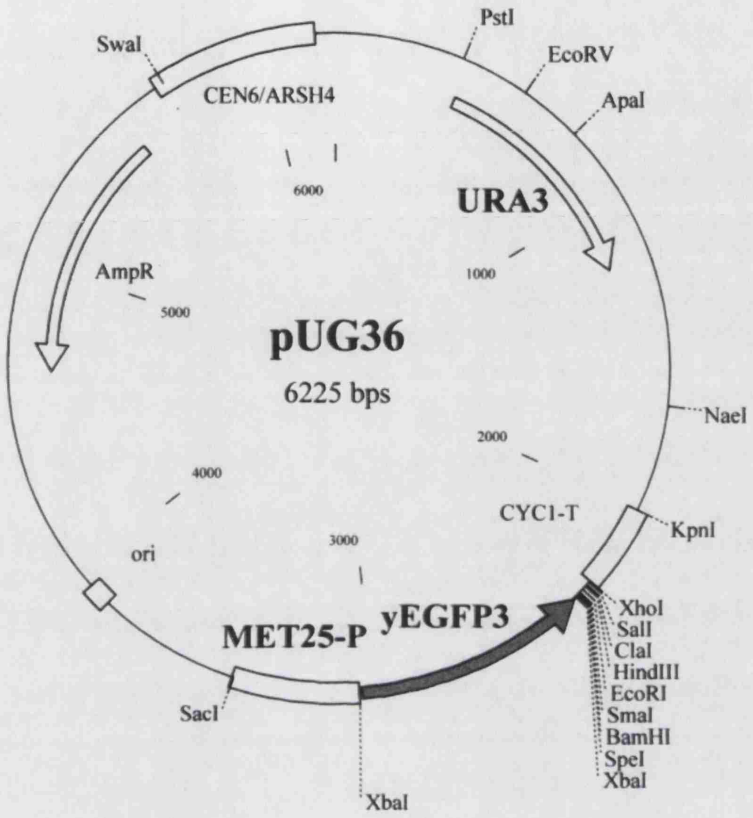
Initially *RLM1* was PCR amplified as an *Nco1/Pst1* fragment (using primers RLM1 F (Nco1) and RLM1 R (Pst1), Table 2.3) and cloned into the pADc vector (Table 2.2). Using this construct as a template, The *RLM1* amino terminal-GAL4p activatory domain fusion (AD-RLM1) was PCR amplified on an *Xba1/Xho1* fragment. The forward primer, AD F (Xba1) (Table 2.3) annealed to the 5' of the GAL4p activatory domain and the reverse primer, RLM1 R (Xho1) (Table 2.3) annealed to the 3' sequence of *RLM1*. The resulting fragment was cloned into the pUT36 vector (Figure 2.1).

2.6.2 *Constitutively-active Mkk1p expression vector, CA-MKK1-pUT36*

As an initial step the *MKK1* gene (containing the S386P mutation) was PCR amplified, from pNV7W-MKK1^{P386} as an *Xba1/Xho1* fragment using primers MKK1 F (Xba1) and MKK1 R (Xho1) (Table 2.3) so that the amplified product could be ligated into the *Xba1/Xho1*-digested expression vector pUT36.

2.6.3 *K54R and T190A, Y192F-SLT2-TOPO vector*

Site-directed mutagenesis was carried out on a TOPO vector (Invitrogen) containing the entire 1.45Kb *SLT2* gene to produce (K54R-SLT2)-TOPO (using primers SLT2 K54R F and SLT2 K54R R, Table 2.3) and (T190A, Y192F-SLT2) TOPO (using primers SLT2 T190A, Y192F F and SLT2 T190A, Y192F R, Table 2.3). These vectors were used as the template source for the construction of AD-K54R Slt2p, AD-T190A, Y192F Slt2p, K54R-6xHis-Slt2p and T190A, Y192F-6xHis-Slt2p.



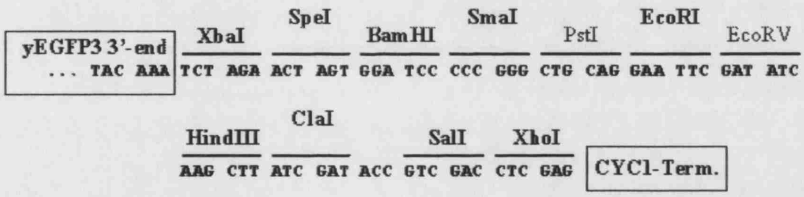


Figure 2.1)

The pUG36 yeast expression vector.

(http://mips.gsf.de/proj/yeast/info/tools/hegemann/gfp.html). The expression vector pUT36 was created from this by excising the yEGFP3 containing *Xba1-Xba1* fragment.

2.6.4 6xHistidine-tagged-Slt2p expression vector, SLT2-6xHIS-pUT36

The *SLT2* carboxy terminal-6xHIS fusion (*SLT2*-6xHIS) was PCR amplified on a *Spe1/Cla1* fragment. The forward primer annealed to the 5' sequence of *SLT2* (SLT2 F (Spe1), Table 2.3) and the reverse primer contained the Gly6xHIS epitope tag (SLT2-6xHIS R (Cla1), Table 2.3). The resultant fragment was cloned into the *Spe1/Cla1* digested pUT36 vector.

2.6.5 ERK2 expression vector, PG1-ERK2

The *ERK2* gene was amplified from ERK2-PGEX-6XT on a *BamH1/Sal1* fragment using primers ERK2 F (BamH1) and ERK2 R (Sal1) (Table 2.3) then ligated into a *BamH1/Sal1* digested vector PG-1.

2.6.6 BMK1 expression vector PG1-BMK1

The *BMK1* gene was amplified from BMK1-pOTB7on a *BamH1/Sal1* fragment using primers BMK1 F (BamH1) and BMK1 R (Sal1) (Table 2.3) then ligated into a *BamH1/Sal1* digested vector PG-1.

Table 2.3) PCR primers used in this study

RLM1 F (Nco1) *(underline=restriction site)*	CATGCCATGGGTAGACGGAAGATTGAA
RLM1 R (Pst1)	TTGGCTGCAGTTATATTTTGCTTGAATTTTTTTCTCC
AD F (Xba1)	CTAGTCTAGAATGGATAAAGCGGAATTAATTCCCGAGCC
RLM1 R (Xho1)	CCGCTCGAGTTATATTTTGCTTGAATTTTTTTCTCC
MKK1 F (Xba1)	CTAGTCTAGAATGGCTTCACTGTTCAGACCC
MKK1 R (Xho1)	CCGCTCGAGTTAATCTTTCCAGCACTTCCT
SLT2 K54R F *(Blue=mutagenic site)*	GATACCACAGTTGCCATCAGGAAAGTGACAAACGTTTTTTC
SLT2 K54R R	GAAAAAACGTTTGTCACTTTCCTGATGGCAACTGTGGTATC
SLT2 T190A, Y192F F	GAAAACAGTCAATTTTTGGCGGAGTTCGTGGCCACTAGATGG
SLT2 T190A, Y192F R	CCATCTAGTGGCCACGAACTCCGCCAAAAATTGACTGTTTTC
SLT2 F (Spe1)	GGACTAGTATGGCTGATAAGATAGAGAGG
SLT2-6xHIS R (Cla1) *(Green=6xHis tag)*	CCATCGATCTAATGATGATGATGATGATGACCAAAATATTTTCTATCTAATC
ERK2 F (BamH1)	CGCGGATCCATGGCGGCGGCGGCGGCGGCGGGCCCGGAGATGGTCCGC
ERK2 R (Sal1)	ACGCGTCGACTTAAGATCTGTATCCTGGCTG
BMK1 F (BamH1)	CGCGGATCCATGGCCGAGCCTCTGAAGGAGGAAGAC
BMK1 R (Sal1)	ACGCGTCGACTCAGGGGTCCTGGAGGTCAGGCAGGTCAGC

2.7 Biochemical techniques

2.7.1 Extraction of total cell protein

Yeast cells were pelleted by centrifugation (3000 rpm, 5min) and the supernatant was discarded. Two volumes of acid washed glass beads (BDH, 40 mesh), were added to the cell pellet. Protein extraction buffer (50mM Tris-HCl[pH8.0], 1mM $MgCl_2$, 2mM EDTA, 1mM DTT, 1mM PMSF, 0.5mM TPCK and 2μg/ml Pepstatin A) was added at a volume sufficient to cover the cell/bead suspension. Cells were disrupted by vortexing for 30 seconds and then chilling on ice for 30 seconds, this procedure was repeated 5 times to ensure complete cell breakage. Beads and cellular debris were removed by centrifugation at (14000rpm, 5min, 4°C) and soluble protein-rich supernatant was removed.

2.7.2 Purification of 6xHistidine-tagged proteins for total cell lysate

Soluble protein-rich extract (obtained as above) containing the 6xHistidine tagged protein of interest was passed through a His-Select column (Sigma) and purified as per manufacturer's instructions.

2.7.3 Determination of protein concentration

Protein concentrations were determined using the BioRad protein determination kit and bovine serum albumin as standard. Both standard and unknown protein concentrations were assayed as by manufacturer's instructions.

2.7.4 Separation of proteins by SDS polyacrylamide gel electrophoresis (PAGE)

Proteins and their subunits can be separated by size using a discontinuous electrophoresis system (Laemmli et al., 1970). Slab gels were cast in the Bio-Rad Mini-Protean-3® system, gel solutions are listed below.

The Laemmli system consists of a stacking gel, containing 4% acrylamide and a resolving gel containing the appropriate percentage of acrylamide for efficient separation of proteins/fragments of interest (ranging from 7-15% acrylamide). Both gels

were cast using a 40% acrylamide:bis-acrylamide stock and polymerised upon addition of 0.05%(v/v) TEMED and 0.7% (w/v) APS.

Prior to loading, protein samples were boiled for 10mins in sample buffer. These samples were loaded into wells formed within the stacking gel, and electrophoresis proceeded at a constant current of 30mA until the appropriate level of protein separation had occurred. Molecular standard markers (Marker 12, SeeBlue2, Invitrogen®) were run beside the samples to indicate protein size. Solutions given below.

2.7.5 SDS-PAGE solutions

30%Acrylamide stock (BDH)	29.2% acrylamide and 0.8%Bis-acrylamide
APS	10% (w/v) solution stored at 4°C
Stacking Gel	125mM Tris-HCL[pH6.8]; 0.1%SDS, 4%polyacrylamide.
Resolving Gel	500mM Tris-HCL[pH8.8]; 0.1%SDS, (x%)polyacrylamide
Electrode buffer[pH 8.3]	0.025M Trizma base; 0.192M glycine and 0.1%SDS
Protein sample buffer	0.125M Tris-HCl[pH6.8]; 10% (v/v) glycerol, 5% (v/v) ßmercaptoethanol; 2%SDS and 0.015% (v/v) bromophenol blue.

2.7.6 Analysis of proteins after SDS-PAGE

After electrophoresis was completed the gel was dismantled and the proteins were visualised by one of the following methods.

2.7.7 Direct staining

Staining with Coomassie blue R-250 allows detection of abundant proteins (1μg or more). Gels were incubated for 1 hour at room temperature, in 0.05% (w/v) Coomassie blue R-250, 50% methanol and 10% acetic acid. Gels were subsequently de-stained by gentle agitation at room temperature in a solution of 10% methanol and 7.5% acetic acid. Destain solutions were periodically changed.

2.7.8 Western blotting

SDS-PAGE separated proteins can be blotted onto a nitrocellulose membranes by means of electrophoresic transfer. The electrophoresed gel, transfer membrane, 6 pieces of Whatmann filter paper were pre-equilibrated in transfer buffer (25mM Tris and 150mM glycine [pH 8.3] with 40% methanol to minimise swelling of the gel during transfer). A gel-blot sandwich was constructed of 3 pieces of filter paper, the gel, the membrane and the remaining sheets of paper. The sandwich was immersed within a buffer-filled Western blotting tank, with gel towards the cathode and membrane towards the anode. The electrophoretic transfer proceeded at 200mA, 10°C, for 2 hours. Efficiency of protein transfer was verified by staining the membrane with Ponceau-S (Sigma). The transient stain was water-soluble and did not affect further analysis of blotted proteins. By running pre-stained protein marker during SDS-PAGE, both the efficiency of transfer and molecular weight of sample proteins, was identified.

2.7.8.1 Immunodetection of proteins on blotted membranes

All immunodetection steps were carried out at room temperature with constant agitation. Tris Buffered Saline + 0.1% Tween (TBS-T) was used as both a base for blocking agent (5% non-fat dried milk powder or 1% Bovine Serum Albumin dissolved in TBS-T), and as between-step washes. Antibodies were diluted in blocking agent and applied to the blot in a final volume of $0.1 ml cm^{-1}$ of membrane.

After Ponceau–S staining, the remaining protein-binding sites on the blot were blocked by incubation in blocking agent for 1 hour. Excess blocking agent was removed with 3 ten minutes washes in TBS-T. Primary antibody was diluted in blocking agent and used to probe the blot for 1 hour. The wash step was repeated before the secondary antibody was diluted in blocking agent and applied to the membrane for a further hour. Again surplus antibody was removed by washing the blot 3 times with TBS-T. Antibody binding was visualised by means of enhance chemiluminessence (ECL), (Amersham). Equal volume of reagents A and B were mixed and applied to the blot for 1 min. The localised fluorescence can be detected by exposure to Fuji X-ray film.

2.8 Yeast two hybrid techniques

2.8.1 Construction of N and C terminal Yeast two-hybrid bait (DBD) fusions

A DNA fragment containing the ORF of interest was generated by two sequential PCR amplifications, essentially as described (Millson et al., 2004; Uetz et al., 2000). The first PCR used primers with that possess 3' sequence homologies to the ORF of interest, but 5' homologies to plasmid pBDC (Millson et al., 2003), while the second PCR used a universal pair of primers. Strain PJ694-α (James et al., 1996), table 2.1) was then transformed with the product of this second PCR and either NcoI- or NruI- digested pBDC, so as to generate N- terminal and C-terminal fusions respectively of the Gal4p DBD to the ORF of interest by homologous recombination within the yeast. Transformants were initially selected by plating on dropout medium (Adams et al., 1997) lacking tryptophan. They were then checked for the presence of the correct fusion by colony PCR (Ling et al., 1995), using the same primers as in the second round of PCR amplification above.

2.8.2 Construction of N and C terminal Yeast two-hybrid activatory (AD) fusions

A DNA fragment containing the ORF of interest was generated by two sequential PCR amplifications, essentially as described (Millson et al., 2003; Uetz et al., 2000). The first PCR used primers with that possess 3' sequence homologies to the ORF of interest, but 5' homologies to plasmid pADC (Millson et al., 2003), while the second PCR used a universal pair of primers. Strain PJ69-4a (James et al., 1996), Table 2.1) was then transformed with the product of this second PCR and added to *PvuII* and *NcoI* digested pADC, so as to generate N- terminal and C-terminal fusions respectively of the Gal4p AD to the ORF of interest by homologous recombination within the yeast. Transformants were initially selected by plating on dropout medium (Adams et al., 1997) lacking leucine. They were then checked for the presence of the correct fusion by colony PCR (Ling et al., 1995)using the same primers as in the second round of PCR amplification above.

2.8.3 Mating of PJ694-a and PJ694-α strains

PJ694-a and PJ694-α cells containing the appropriate AD and BD-fusions were incubated overnight together in YPD and then placed onto media lacking leucine and tryptophan. Resultant colonies were restreaked and used in the β-Galactosidase assay (see below).

2.8.4 Assay of β-Galactosidase in yeast

Yeast cells to be assayed were grown from a culture inoculated overnight in 2x5ml selective media, then placed into 200ml media of the appropriate selective media and grown until they reached an $O.D_{600}$ of around 0.8. 20ml aliquots of the culture were taken and placed in the stress concerned for one hour. After this time, the O.D. of each 20ml sample was measured at 600nm. Each sample was spun down and resuspended in 1ml of Z buffer (solution given below). 3 drops of chloroform and 2 drops of 0.1% SDS were added, and each sample was vortexed for one minute. After incubation at 28°C for 15 minutes, 200μl of ONPG solution (4mg/ml ONPG in Z buffer) was added and the reaction left for enough time until a faint yellow colour was observed. At this point, 500μl of 1M Sodium Carbonate was added to terminate the reaction. After centrifugation at 10,000rpm for 10 mins, the $O.D_{420}$ of each sample was taken. The β-galactosidase value was calculated via the following equation:

Value (mU)=1000*(OD_{420}/(OD_{600}*reaction volume in ml*time reaction left in mins.))

Z-Buffer: 16.1g $Na_2HPO_4.7H_2O$, 5.5g $NaH_2PO_4.H_2O$, 0.75g KCl, 0.246g $MgSO_4.7H_20$, 2.7ml β-Mercaptoethanol (made up to 1l with distilled H_2O and adjusted to pH 7 via 5M NaOH)

3 The link between HSF regulation and the yeast cell integrity pathway

3.1 Introduction

Mammalian, fly and plant HSFs possess just a single *trans*-activation domain. In contrast, two quite separable *trans*-activation domains are present on the HSFs of *S. cerevisiae* and *K. lactis*: an amino-terminal activation domain (in *S. cerevisiae* HSF residues 1 to 172) and a carboxyl-terminal activation (CTA) domain (in *S. cerevisiae* HSF residues 584 to 833(Nieto-Sotelo et al., 1990; Sorger, 1990). The CTA domain on *S. cerevisiae* HSF is nonessential, though its loss compromises the induction of certain genes, but not others, in response to heat shock (Sorger, 1990; Tamai et al., 1994). Thus this CTA domain is largely dispensable for heat activation of several Hsp70 genes (*SSA1*, *SSA3* and *SSA4* (Tamai et al., 1994; Young et al., 2003)), but its loss drastically reduces the induction of the *CUP1* gene by heat, oxidative stress and glucose starvation (Liu and Thiele, 1996; Tamai et al., 1994). In addition both basal and heat-induced expressions of the two *S. cerevisiae* Hsp90 genes, *HSP82* and *HSC82*, are markedly reduced with loss of this HSF CTA domain (Morano et al., 1999). It has been suggested that these disparate influences of the CTA on the heat-induced expressions of different genes might reflect differences in the architecture of the heat shock element (HSE), the alternating repeats of the nGAAn motif that constitute the binding site on DNA for HSF (Santoro et al., 1998).

Loss of the CTA domain on *S. cerevisiae* HSF (the *HSF(1-583)* mutant) generates lower *sustained* HSF activity at all temperatures, even though it elevates HSF levels in cell extracts (Bonner et al., 2000). The cells also become temperature sensitive (*ts*), displaying no growth above about 35°C (Chen et al., 1993; Jakobsen and Pelham, 1991; Nieto-Sotelo et al., 1990; Sorger, 1990). Genes under the control of this HSF CTA domain must therefore be essential for yeast growth at high temperatures. Almost a full capacity for high temperature growth can be restored to the *HSF(1-583)* mutant cells with overexpression of the Hsp82 isoform of the yeast Hsp90 chaperone (though not an

overexpression of the 97% similar Hsc82 (Morano et al., 1999). The requirement for CTA domain-directed gene expression at high temperatures may therefore be the specific need to induce a high level of Hsp82, the strongly heat-inducible isoform of Hsp90.

Morano (Morano et al., 1999) concluded that the *HSF(1-583)* mutant cells were undergoing G2 cell cycle arrest at high temperature. However, we had observed that the *HSF(1-583)* mutant cells became very swollen at temperatures greater than 34°C, a phenotype associated with an inability to maintain cellular integrity. This Chapter describes a study that revealed the *HSF(1-583)* mutant is defective in maintenance of cell integrity at high temperatures, also that the HSF CTA domain is required for the reinforcement of Rlm1p-mediated cell integrity gene expression during high temperature growth. Both the heat shock response and the cell integrity pathway are activated by heat shock, but this is the first evidence that HSF activity indirectly controls heat activation of cell integrity gene expression, mainly through effects on Hsp82 levels.

3.2 Results

3.2.1 Cells lacking the HSF CTA domain display an osmoremedial ts phenotype

The *ts* phenotype that results from loss of the CTA domain on HSF (the *S. cerevisiae HSF(1-583)* mutant: Table 2.1) is rescued with a non-HSF directed overexpression of the heat-inducible Hsp82 isoform of yeast Hsp90 but, remarkably, not a similar overexpression of the almost identical, constitutively-expressed Hsc82 isoform (Morano et al., 1999) (see Introduction). As shown in Figure 3.1a, high temperature growth can also be restored to the *HSF(1-583)* cells with osmotic stabilisation of the medium (Figure 3.1a). The CTA domain of HSF is therefore nonessential for high temperature growth when cells are osmotically stabilized. We also observed *HSF(1-583)* mutant cells to become enlarged and swollen 1-2h after a shift from 30°C to 39°C in the absence, though not the presence, of osmotic stabilisation (Figure 3.1b).

3.2.2 Osmotic stabilisation of HSF(1-583) cells does not affect either HSF activity or Hsp82 levels

To see whether osmotic-stabilisation was affecting either the basal or the heat induced activities of the wild type or the CTA domain-deficient HSF, we measured HSF activity in strains NSY-A and NSY-B (Table 2.1). At all time points measured, there was no detectable effect of the presence of 1.2M sorbitol on a *LacZ* reporter gene under HSE element control (HSE2-LacZ (Sorger and Pelham, 1987)) (Figure 3.2).

Osmotic-stabilisation was also not rescuing the defective heat-induction of Hsp90 levels in the *HSF(1-583)* mutant (Figure 3.3). We considered whether these effects might be due to the presence of *ssd1-3*, a mutation that sensitises strains to stress, and which is present in these strains of W303 genetic background (Costigan et al., 1992). A HSF CTA-deficient strain in a genetic background that lacks *ssd1-3* was therefore generated (Table 2.1). An osmoremedial *ts* phenotype was still present in the absence of *ssd1-3* (data not shown).

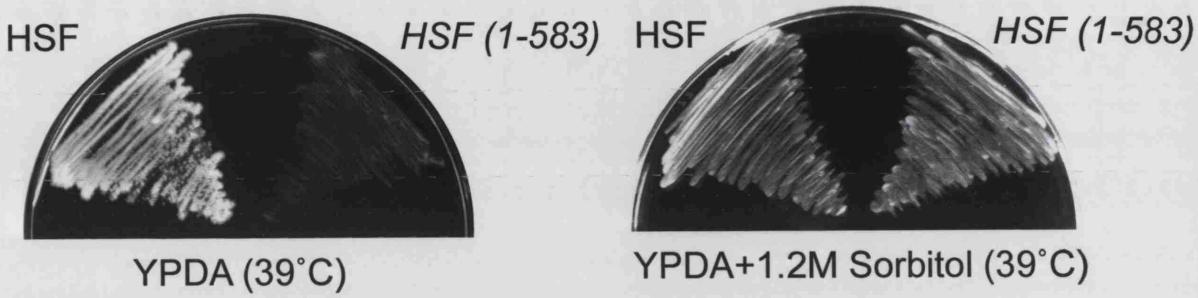
Figure 3.1a)

Osmotic stabilization rescues high temperature growth of a *S. cerevisiae* strain NSY-B, *ts* due to the loss of the HSF CTA, (Table 2.1). The corresponding wild-type strain (NSY-A) is designated HSF. Plates were photographed after 4d.

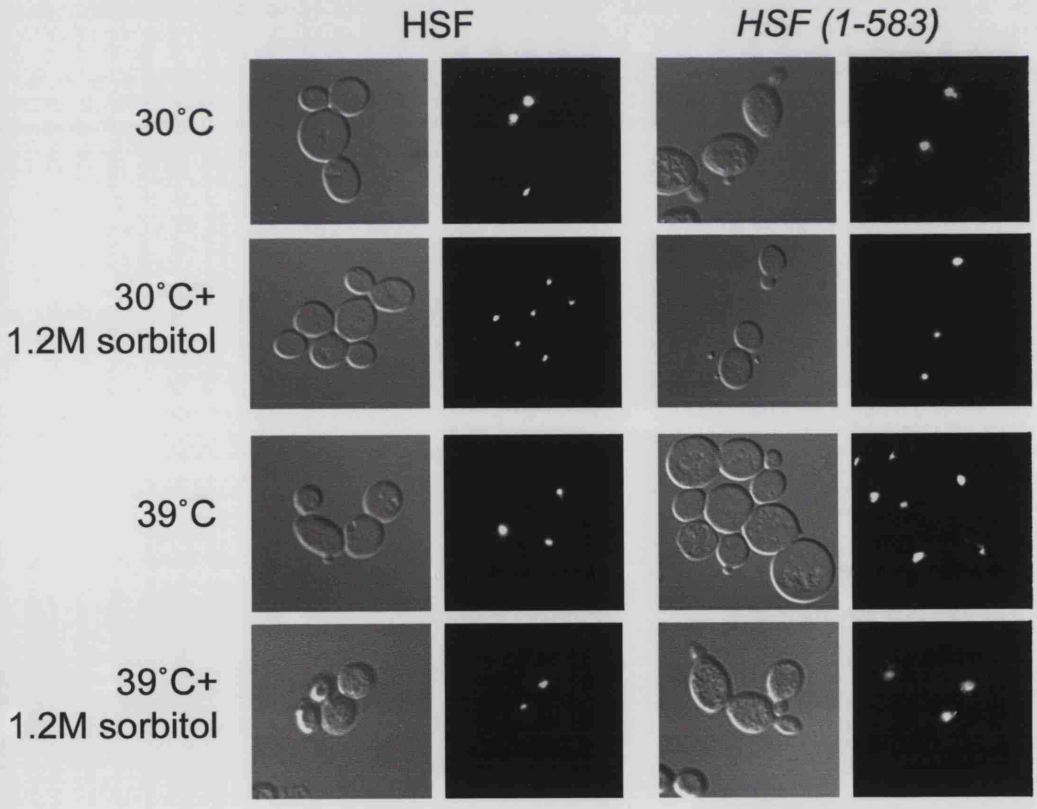
Figure 3.1b)

Phase-contrast and nuclear (DAPI) stained images of unstressed and heat shocked (1h 39°C) cells (of the HSF and *HSF(1-583)* strains, maintained in the presence and absence of osmotic stabilization); x100 magnification.

a)


HSF
HSF (1-583)
YPDA (39˚C)
HSF
HSF (1-583)
YPDA+1.2M Sorbitol (39˚C)

b)


HSF
HSF (1-583)
30˚C
30˚C+
1.2M sorbitol
39˚C
39˚C+
1.2M sorbitol

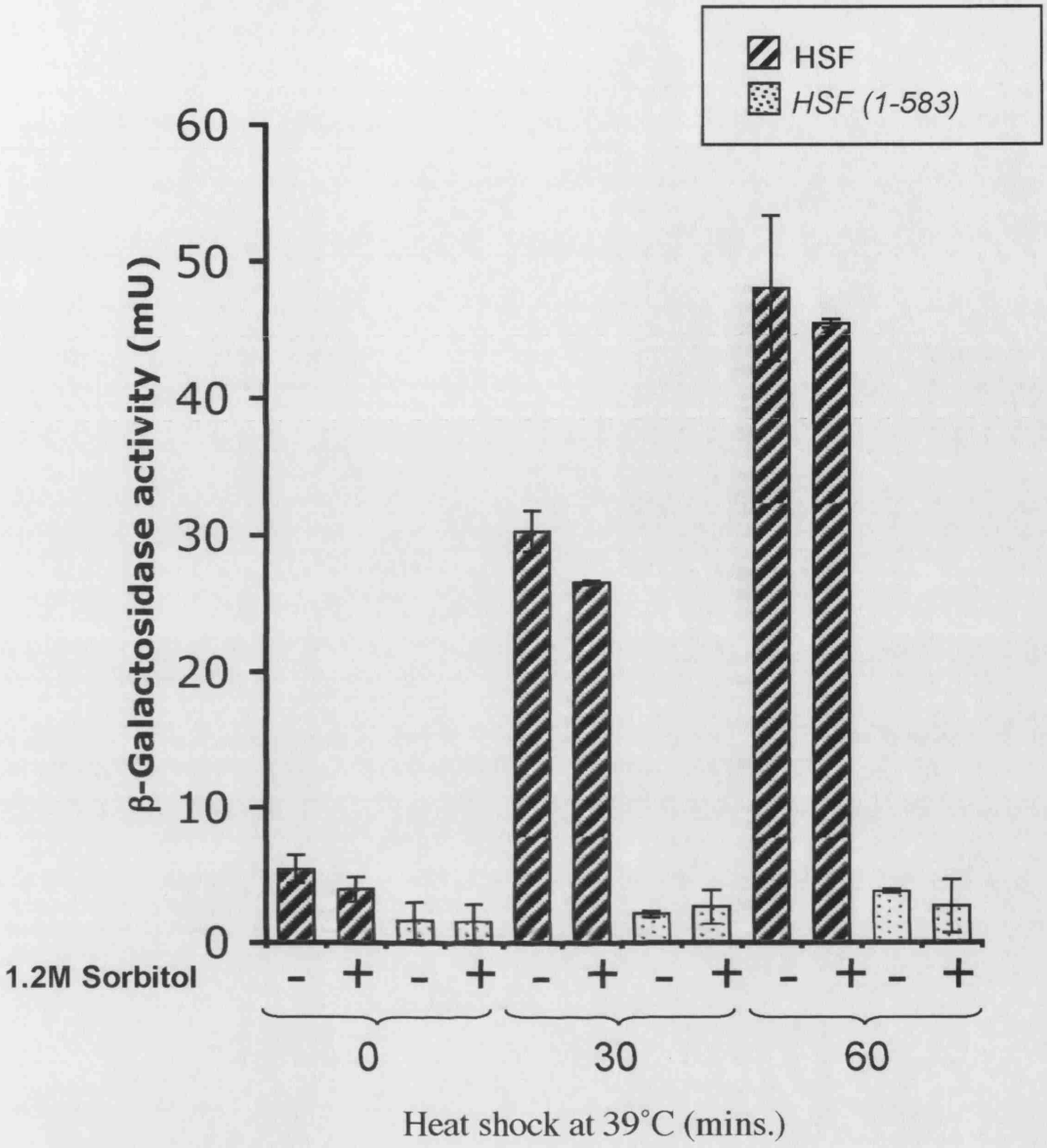


Figure 3.2)

Osmotic stabilisation does not restore heat induction of an HSE-*LacZ* reporter gene in the *HSF(1-583)* mutant. NSY-A (*HSF* wild type) and NSY-B (*HSF(1-583)*) cells were grown to mid-log phase in media lacking uracil in the presence or absence of 1.2M sorbitol. The cells were then exposed to 39°C for either 0, 30 or 60 mins and HSE-*LacZ* activity was measured. Data shown is the mean and SD of three separate experiments.

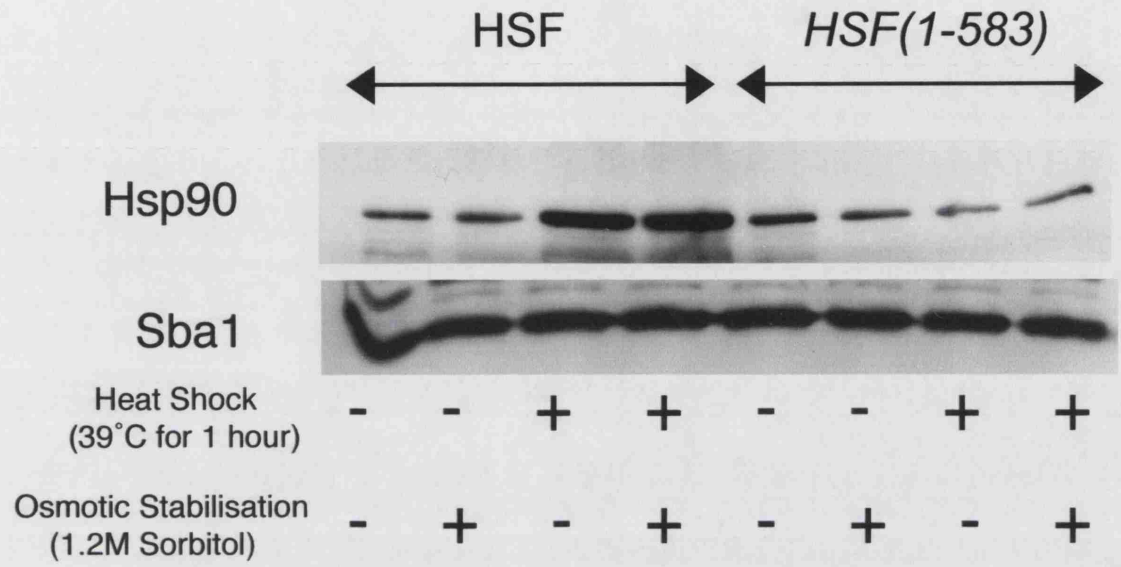


Figure 3.3)

Osmotic stabilisation does not rescue heat inducibility of Hsp90 in *HSF(1-583)* cells. NSY-A (HSF) and NSY-B (*HSF(1-583)*) cells were grown to mid-log then placed at either 30°C or 39°C for 1 hour in the presence or absence of 1.2M sorbitol. 10μg of total cell extract was analysed on a 10% SDS-PAGE gel. Proteins were transferred to nitrocellulose and total Hsp90 and Sba1p levels were measured using rabbit polyclonal antisera.

3.2.3 *The effect of caffeine, an activator of the yeast cell integrity pathway on the HSF(1-583) mutant*

The *HSF(1-583)* mutant was also slightly caffeine-sensitive at its highest temperatures of growth (30-35°C) (Figure 3.4a), even though millimolar caffeine levels have no appreciable effects on HSF activity (Figure 3.4b). Rescue of high temperature growth by osmotic stabilisation and caffeine sensitivity at lower temperatures are phenotypes characteristic of yeast mutants defective in cell wall maintenance, especially those defective in the Slt2p MAP kinase branch of Pkc1p-mediated signalling (Gustin et al., 1998; Martin et al., 2000). This study therefore investigated if the loss of the CTA domain on HSF might be causing defective cell integrity signalling at high, supraoptimal temperatures of growth. In addition, because this *HSF(1-583)* defect is suppressed by Hsp82 overexpression (Morano et al., 1999), investigations were conducted into whether the partial loss of Hsp82 function in yeast might also generate similar defects in cell integrity signalling (discussed in Chapter 4).

3.2.4 *The HSF(1-583) mutant is defective in stress-induction of Rlm1p activity*

It appears Rlm1p is the main trans-activator of cell wall genes in yeast and activated primarily by Slt2p (Jung and Levin, 1999). Its activity is readily monitored using a reporter gene for Rlm1p activity (*YIL117c-LacZ* (Jung et al., 2002)). This *YIL117c-LacZ* construct was transformed into wild type (NSY-A) and *HSF(1-583)* mutant (NSY-B) strains. Basal Rlm1p activity and Rlm1p activity following heat shock at 39°C or the addition of caffeine to the media were then measured. *YIL117c-LacZ* expression measurements in *HSF(1-583)* cells (Figure 3.5) revealed that the loss of the HSF CTA domain had a minimal effect over basal Rlm1p activity in cells in growth at 25°C, but largely abolished any heat- or caffeine-induction of this Rlm1p activity. This defect in the stress-induction of Rlm1p activity may be due to the low Hsp82 level in this mutant, since a non-HSF-directed Hsp82 overexpression can restore high temperature growth to these *HSF(1-583)* mutant cells(Morano et al., 1999) (see Introduction). This study therefore investigated whether non-HSF-directed Hsp82 overexpression could also

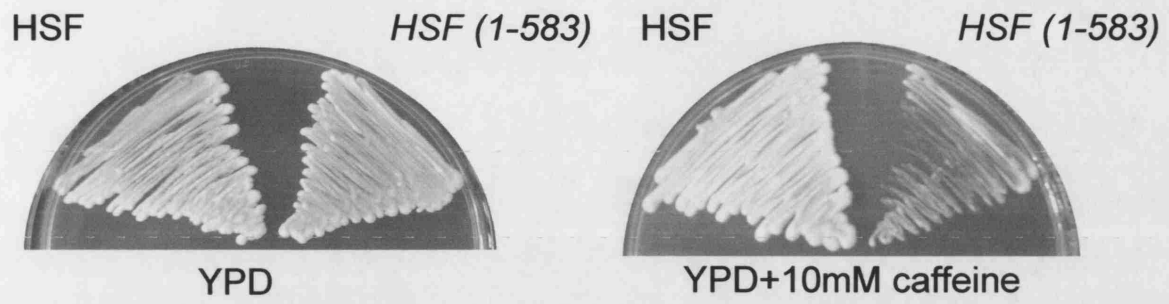
Figure 3.4a)

Caffeine sensitivity of HSF and *HSF(1-583)*. Cells were streaked on YPD in the presence or absence of 10mM caffeine. Plates were photographed after 4 d at 30°C.

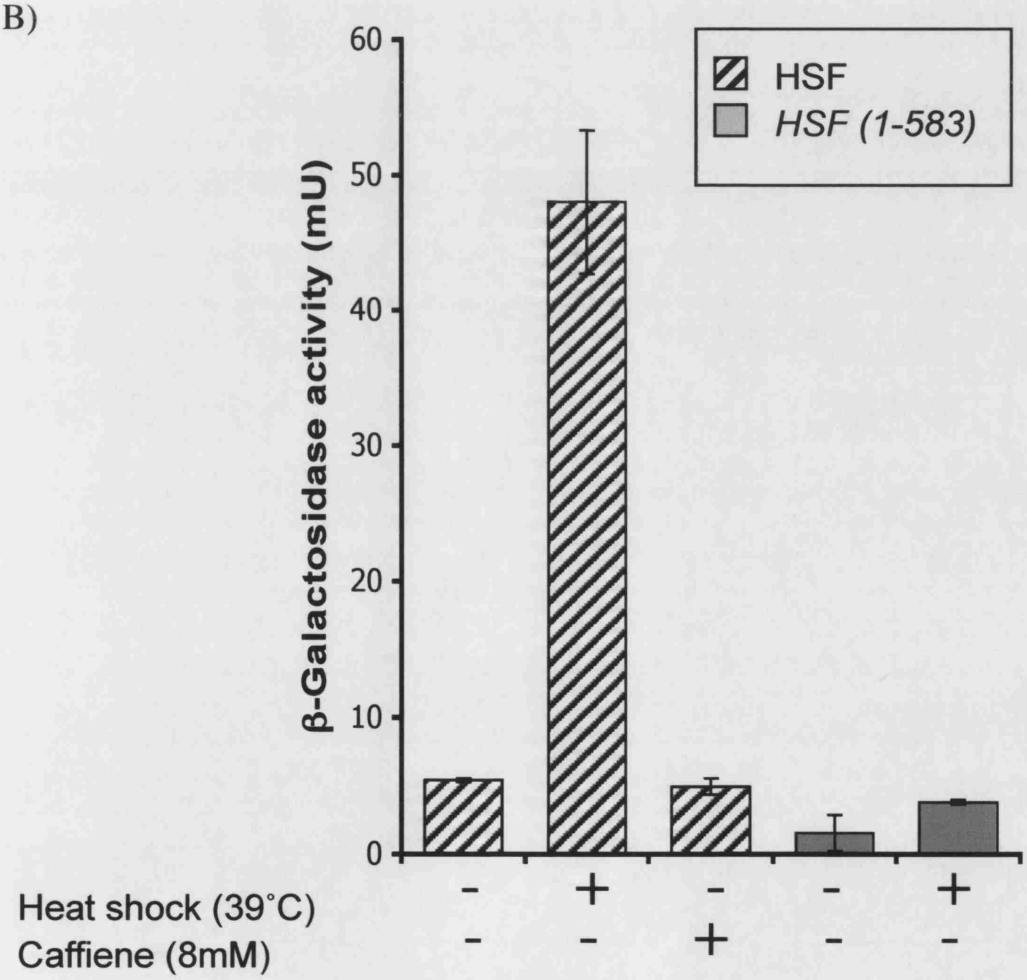
Figure 3.4b)

HSF activity is not induced by caffeine. Cells were grown to mid log as in Fig. 3.2 and exposed to 8mM caffeine for 1 h. β-Galactosidase activity was measured as previously (mean and SD of three experiments are shown).

A)


HSF
HSF (1-583)
YPD
HSF
HSF (1-583)
YPD+10mM caffeine

B)


β-Galactosidase activity (mU)
60
50
40
30
20
10
0
HSF
HSF (1-583)
Heat shock (39°C)
Caffiene (8mM)
- + - - +
- - + - -

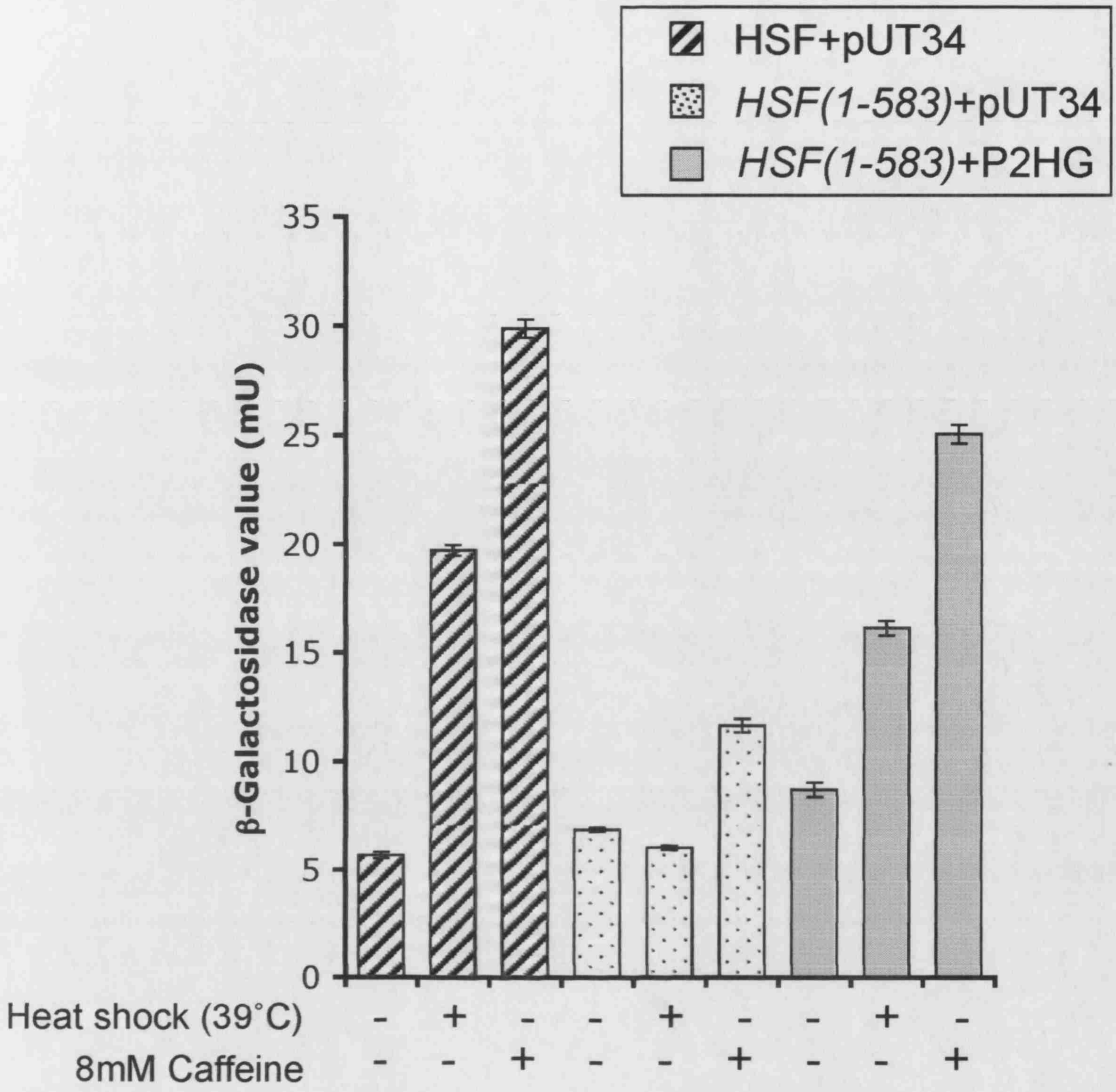


Figure 3.5)

A non HSF-directed Hsp82 overexpression substantially restored stress induction of Rlm1p activity in the *HSF(1-583)* mutant. Strains were grown to mid log phase on media lacking histidine and uracil, and cells were treated with either 39°C for 1 h or 8mM caffeine for 1 h. Rlm1p-activity was then measured using a *YIL117c-LacZ* reporter plasmid (Jung et al 2002). Results are the mean and SD of three separate experiments.

restore the stress-induction of Rlm1p activity to these *HSF(1-583)* mutant cells. Consistent with earlier findings (Morano et al., 1999), a *GPD1* promoter-directed overexpression of Hsp82 substantially rescued both high temperature growth (Figure 3.6a, 3.6b) and heat and caffeine induction of Rlm1p activity (Figure 3.5) in the *HSF(1-583)* mutant. It is therefore primarily the low Hsp82 level that, in cells expressing HSF lacking the CTA domain, that is compromising the stress activation of Rlm1p.

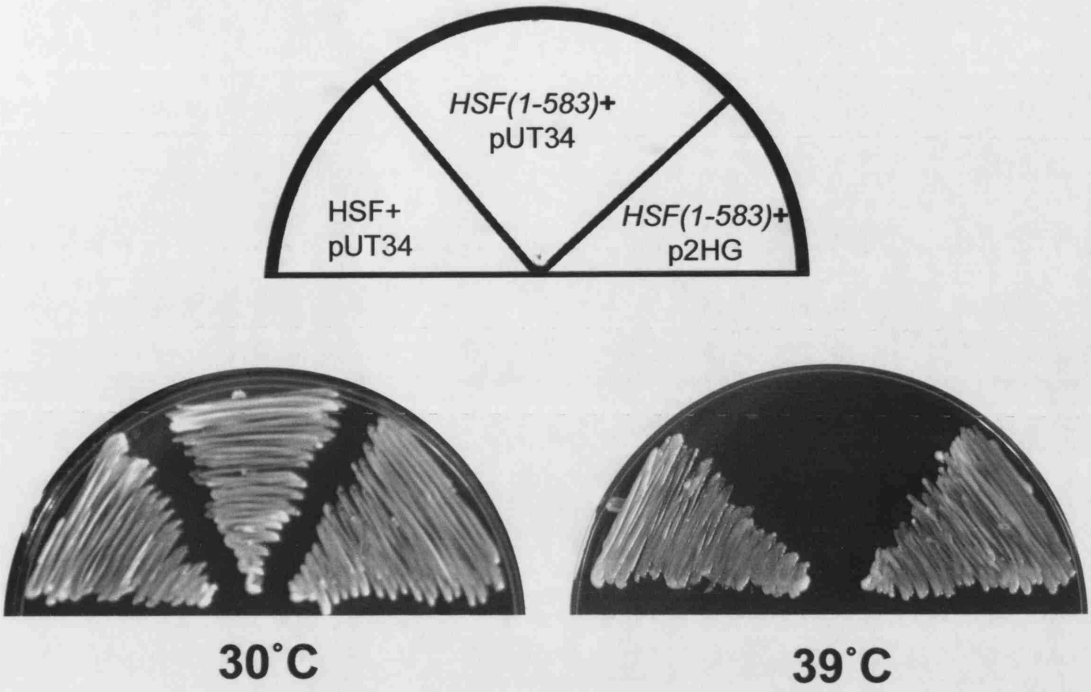
3.2.5 Inhibition of Hsp90 function by radicicol result in abolishment of Rlm1p activity

Hsp90-dependence of Rlm1p activation was demonstrated using the Hsp90-inhibitor radicicol. Even small quantities of the drug resulted in loss of Rlm1p activity under all conditions (Figure 3.7). However such drug inhibitor experiments, though showing the Hsp90 dependence of cell integrity signalling, do not readily identify which step(s) of this signalling require the chaperone function.

3.2.6 The MAP kinase Slt2p is dually phosphorylated in a stress dependant manner in the HSF CTA mutant

Heat stress activates cell integrity signalling in yeast. This occurs mainly through a stimulation of the Slt2p (Mpk1p) MAP kinase branch of protein kinase C (Pkc1p)-mediated signalling (Kamada et al., 1995). Nevertheless the general stress response mediated by Msn2/4p, and a Ca^{2+}-calcineurin response mediated through Crz1p, also contribute to reconfiguring of global gene expression that allows reinforcement of cell wall integrity during growth at high temperatures (Lagorce et al., 2003). Cell wall integrity is monitored continuously by Rho1p, a GTPase that acts both as a regulatory subunit of the 1,3-β-glucan synthase complex and an activator of Pkc1p (see (Gustin et al., 1998; Jung and Levin, 1999; Kamada et al., 1996; Nonaka et al., 1995) for literature). Pkc1p in turn controls the cell integrity MAP kinase cascade, composed of a MAP kinase kinase kinase (Bck1p; (Levin et al., 1994)), a pair of redundant MAP kinase kinases (Mkk1/2p; (Irie et al., 1993)) and the Slt2p stress-activated MAP kinase (see introduction).

a)



b)

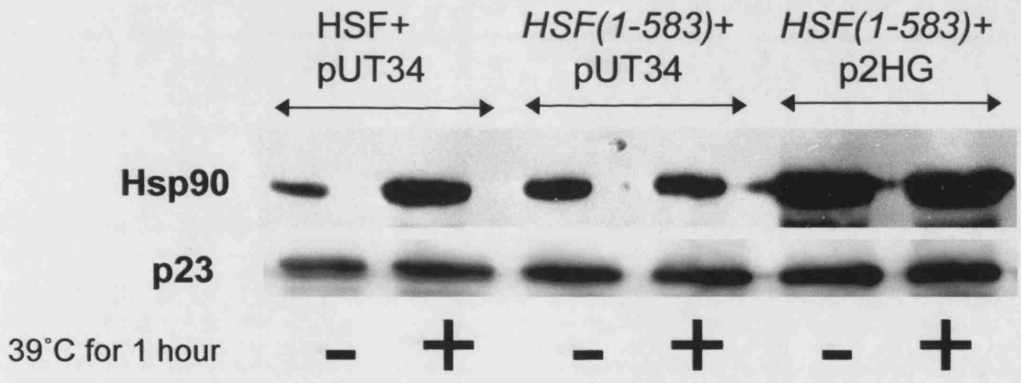


Figure 3.6)

a) *GPD1* promoter-directed overexpression of Hsp82 substantially rescues high temperature growth of the *HSF (1-583)* mutant. b) The cells containing the p2HG plasmid express Hsp82p at a constitutively high level.

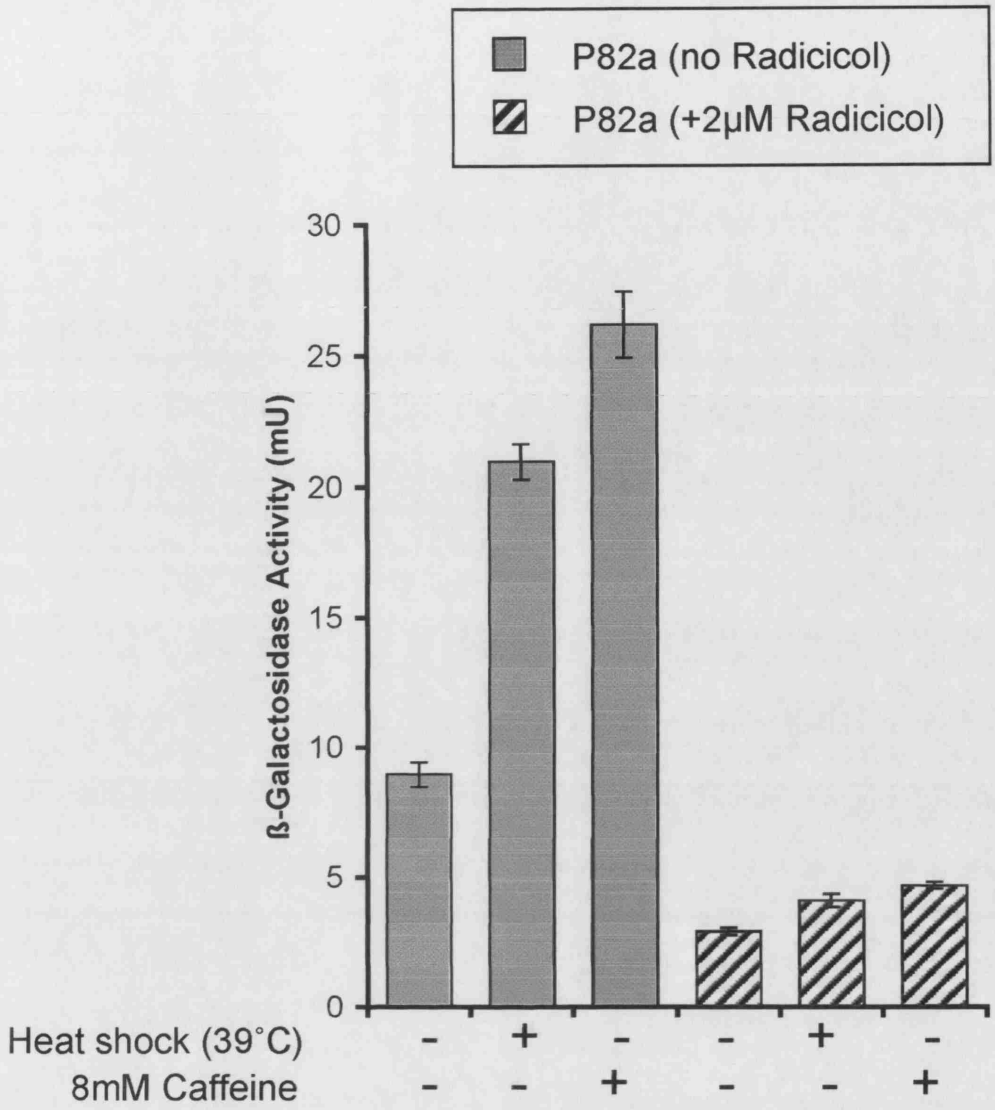


Figure 3.7)

The effect of the Hsp90 inhibitor radicicol on heat and caffeine stress activation of *YIL117c*1-*LacZ* activity in wild type cells. Cells were grown to mid-log at 21°C and incubated for a further 1 h under stress conditions of 39°C or the presence of 8mM caffeine. Where present, 2μM radicicol was added immediately prior to the stress. Results are the mean and SD of three separate experiments.

As described above, cells of the *HSF(1-583)* mutant display an osmoremedial *ts* phenotype (Figure 3.1) similar to that of mutants of cell integrity MAP kinase signalling. As with other kinases of the MAP kinase family, activity of Slt2p depends on the upstream Mkk1/2p catalysing dual threonine/tyrosine phosphorylation at the TXY motif in the activation loop characteristic to MAP kinases (Canagarajah et al., 1997). Heat and caffeine are two of the inducers of this dually phosphorylated, active Slt2p in yeast (Kamada et al., 1995; Martin et al., 2000). We therefore analysed whether the *HSF(1-583)* allele alters Slt2p phosphorylation state in unstressed, heat-shocked and caffeine-stressed cells. Extracts were prepared from NSY-A (HSF) and NSY-B (*HSF(1-583)*) cells (Table 2.1), either in growth at 25°C (unstressed), treated with 8mM caffeine for 2h at 25°C, or heat shocked from 25°C to 39°C for 1h. Levels of both total Slt2p and phosphorylated Slt2p in these extracts were then analysed.

As shown in Figure 3.8, the *HSF(1-583)* mutant was not defective in the normal increases in Slt2p phosphorylation in response to caffeine- or heat-stress. A loss of the HSF CTA domain is not therefore causing loss of Slt2p phosphorylation in response to stress activation of the Slt2p branch of Pkc1p-mediated signalling (Figure 3.8). Instead there was a raised level of this dually Thr^{190}/Tyr^{192}-phosphorylated Slt2p in unstressed *HSF(1-583)* mutant cells growing at 25°C (Figure 3.8). This may be a further indication that the mutant possesses a weakened cell wall, one of the signals for Slt2p pathway activation being the Rho1p-mediated sensing of weakening of the cell wall architecture (see above). Alternatively, it could reflect *HSF(1-583)* affecting the phosphatase action on the dually phosphorylated Slt2p. Levels of *SLT2* gene transcripts are known to increase in heat stressed cells (Hahn and Thiele, 2002), but we were unable to demonstrate a corresponding increase in Slt2p protein levels (Figure 3.8 and data not shown).

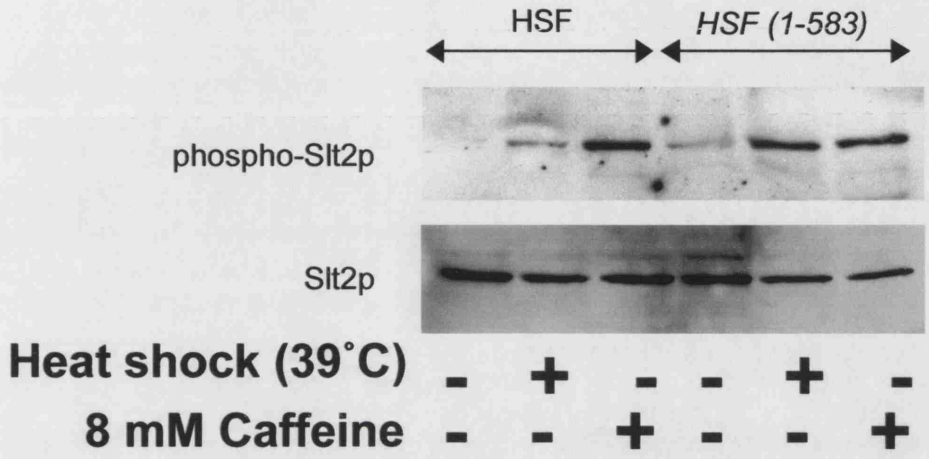


Figure 3.8)

Effects of the *HSF (1-583)* mutation on Slt2p phosphorylation state in cells in growth at 25˚C (unstressed), heat shocked from 25˚C to 39˚C for 1h or treated with 8mM caffeine for 2h at 25˚C.

3.2.7 Expression of a constitutively active Rlm1p suppresses the stress-sensitive phenotype of HSF(1-583)

The above findings suggested that Slt2p was being phosphorylated in the *HSF (1-583)* mutant in response to Pkc1p pathway activation, but that this activatory signal was not being transduced to Rlm1p. Watanabe et al (1995) described how fusion of the Gal4p activatory domain to the N-terminus of Rlm1p resulted in a constitutively active form of this transcription factor, a form that could suppress the *ts* and caffeine sensitive phenotypes of mutants defective in the Slt2p MAP kinase branch of Pkc1p-mediated signalling.

A vector for a *MET25* promoter-driven expression of this AD-Rlm1p fusion was constructed (Section 2.6.1). Induction of this fusion, achieved by depriving the cells of methionine, was alone sufficient to suppress the *ts* phenotype of *HSF(1-583)* cells (Figure 3.9) The major defect preventing high temperature growth of these cells is therefore lack of Rlm1p activity. The HSF mutant is only defective in Rlm1p activity under conditions of stress (Figure 3.5) and caffeine sensitive at its highest temperatures of growth (30-35°C; Figure 3.4a)).

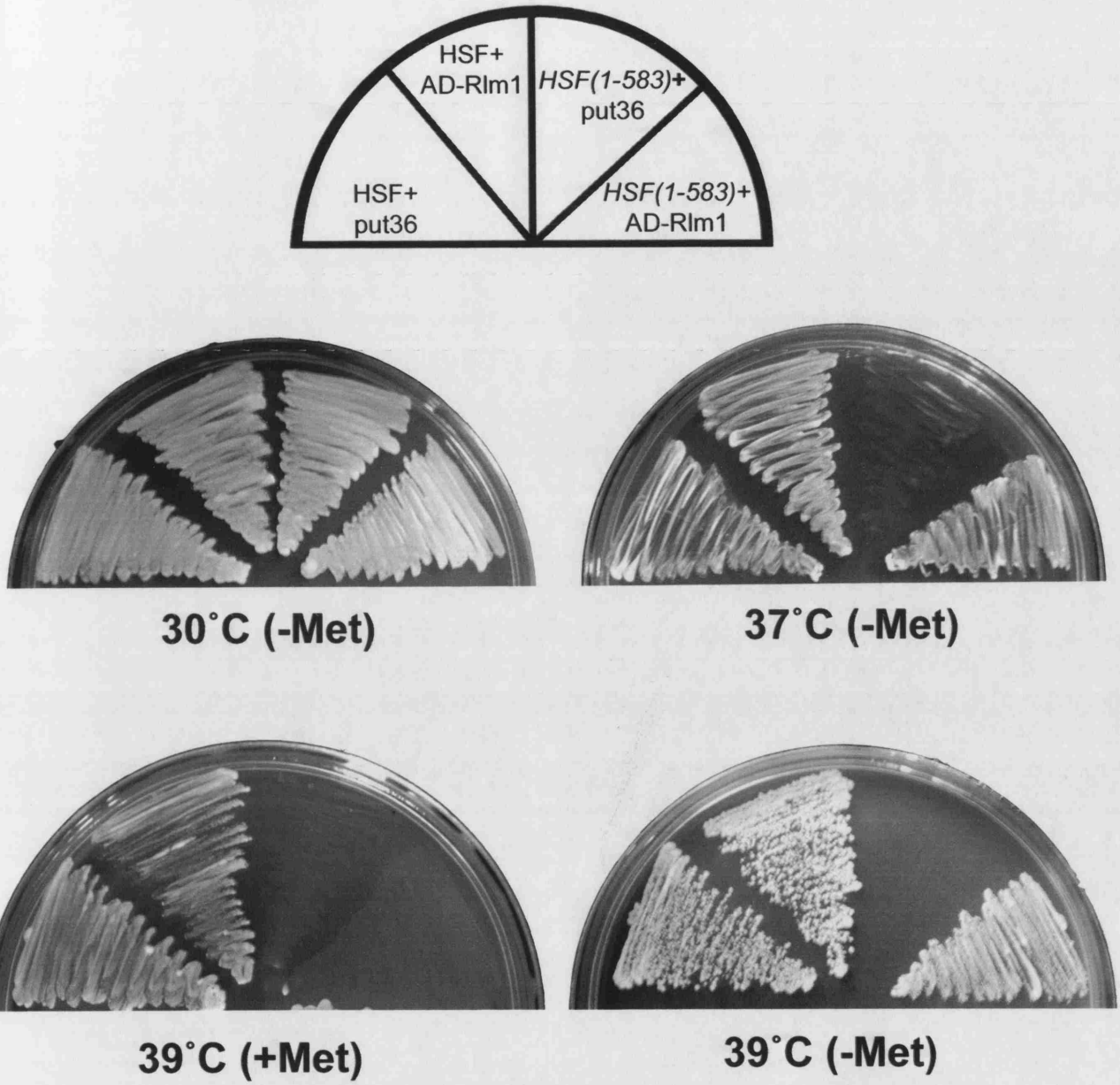


Fig. 3.9)

Induction of a constitutively-active AD-Rlm1p fusion, induced by removal of methionine from the medium, efficiently suppressed the *ts* phenotype of *HSF(1-583)* cells. Plates were photographed after 4d.

3.3 Conclusion

The results in this chapter on the *HSF(1-583)* / *S. cerevisiae* mutant reveal that the HSF CTA regulates, indirectly through the effect on Hsp90 level, a completely different transcription factor, the Rlm1p regulator of cell integrity genes. Only stress-induction of the latter transcription factor is subject to this indirect regulation. The *ts* phenotype of *HSF(1-583)* was suppressed by osmotic stabilisation (Figure 3.1), or by Hsp82 overexpression (Figure 3.6a). The former indicates a clear defect in cell integrity as only these class of mutants can grow at high temperatures in the presence of osmotic stabilisation. This is in contrast to cell-cycle defective mutants which arrest at non-permissive temperatures as swollen, unbudded cells even in the presence of osmotic stabilisation. The *HSF(1-583) ts* phenotype is due to defective Rlm1p activation by stress (Figures 3.5, 3.9). This mutant exhibits no defect in Slt2p phosphorylation by stresses that activate Pkc1p-mediated signalling (Figure 3.8). MAP kinases are known to work by becoming dually phosphorylated in response to stress, dimerising and then activating downstream targets (see Introduction). In The *HSF(1-583)* cells there appears to be a defect in transduction of the stress-induced signal to the Rlm1p target of this phosphorylated Slt2p (Figure 3.5 and data shown later in Chapter 4), partially rescued by Hsp82 overexpression. Chapter 4 provides further evidence that Hsp90 function is needed for the phosphorylated Slt2p to activate its downstream target, Rlm1p.

4 Dissecting the role of Hsp82 on the cell integrity pathway using hsp82 mutants

4.1 Introduction

In 1995 Nathan and Lindquist reported the isolation of an isogenic set of *S. cerevisiae* mutants *ts* for growth at 37°C due to point mutations in Hsp82, their sole form of Hsp90 chaperone (Nathan and Lindquist, 1995). These mutants express Hsp82 to similar levels (Piper et al., 2003a). The mutants could be separated into two distinct classes. One, G170D, had a classic *ts* phenotype, with nearly wild-type activity at 25°C but a distinct loss of function at 34°C. The remaining seven mutants in contrast, caused variable loss of Hsp90 function and may have been *ts* because they did not provide the higher Hsp90 activity required for growth at high temperatures. The mutants are a useful tool for studying Hsp82 function since they occur throughout the protein; T22I, A41V, G81S, T1011 and G170D all occur in the N-terminal domain essential for the binding and hydrolysis of ATP; G313S, E381K and A587T occur in the mid and C-terminal domains, responsible for client binding and constitutive dimerisation of the Hsp82 molecule respectively. These mutants have been used to demonstrate the effect of loss of Hsp82 function on target client proteins, such as the glucocorticoid receptor and v-src (Nathan and Lindquist, 1995; Nathan et al., 1997). It is only relatively recently that the effects of these mutations on the Hsp82 ATPase reaction have been uncovered. In 2000, a study by Prodromou (Prodromou et al., 2000), demonstrated that some of these mutations affect the rate of ATP hydrolysis. The purified T22I mutant Hsp82 protein displayed an intrinsic ATPase rate of around five times that of the WT protein, whereas two other mutant forms of this protein, A41V and T101I showed almost undetectable levels of ATPase activity. Observing the phenotypic effects when expressing these mutant forms of Hsp82 as the sole Hsp90 of the cell could potentially lead to the discovery of novel, model client proteins. Although a potentially risky strategy (due to the large number of client proteins that could be affected by such mutations) it was decided to see if any of the Lindquist mutants displayed a cell integrity defect similar to that of the HSF mutant *HSF (1-583).*

4.2 Results

4.2.1 Many Hsp90 point mutations that cause temperature-sensitivity of yeast at 37-39°C generate caffeine sensitivity at lower growth temperatures.

Investigating these eight *hsp82* mutants in more detail, we found the *ts* phenotypes of no less than six of them were found to be osmoremedial (Figures 4.1, 4.2). In only two of the mutants (A41V*hsp82*; G170D*hsp82*) was the high temperature growth not restored by the presence of 1.2M sorbitol (Figure 4.2). Clearly the other six mutants are able to progress through the cell cycle at high temperatures when their cells are osmotically stabilized (Figure 4.2).

Several of these mutants were also found to be caffeine sensitive during growth at room temperature (Figure 4.3). T22I *hsp82*, the mutant of the set that displays the strongest ***ts*** phenotype (Figure 4.1) is also the mutant that is the most caffeine sensitive; while certain other mutants (T101I*hsp82*, and E381K*hsp82*) display sensitivities to caffeine that are intermediate between that of this T22I*hsp82* and the p82a cells that express the wild type Hsp82 (Figure 4.3). Nevertheless despite the T22I*hsp82* mutant being extremely sensitive to both high temperature and caffeine (Figures 4.1 4.3), it is still able to grow at 39°C (Figure 4.2) when the cells are osmotically stabilized. Its caffeine sensitivity is also osmoremedial (not shown).

It is interesting to note that those Hsp82 mutations that generate caffeine-sensitivity at low temperatures (T22I, T101I, G170D and E381K; Figure 4.3) are a discrete *subset* of those displaying sorbitol-remedial ***ts*** growth (T22I, G81S, T101I, G313S, E381K, A587T; Figure 4.2). The former mutations may cause, to variable extents, defects in cell integrity at all temperatures, while G81S, G313S and A587T may cause a defective integrity only at higher temperatures of growth.

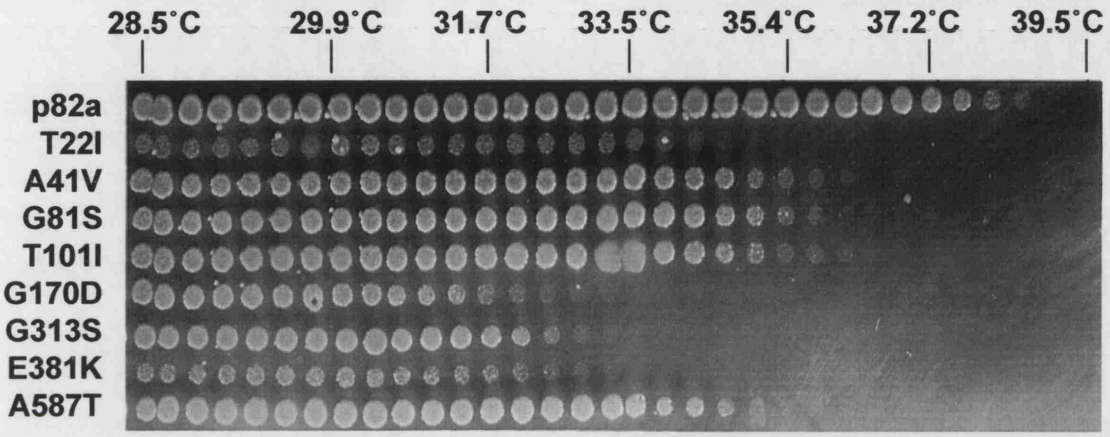


Figure 4.1)

Maximal growth temperature of the *ts hsp82* mutants from the Nathan & Lindquist study (1995). Mid-log-phase cultures of wild type (p82a) and *hsp82* mutant strains grown at 25°C were spotted onto an agar slab containing YPD medium. A temperature gradient was applied to the slab, and the cells were allowed to grow for 40 h. Maximum growth temperatures varied by less than 0.28°C in two independent experiments. (Reproduced from Nathan and Lindquist 1995).

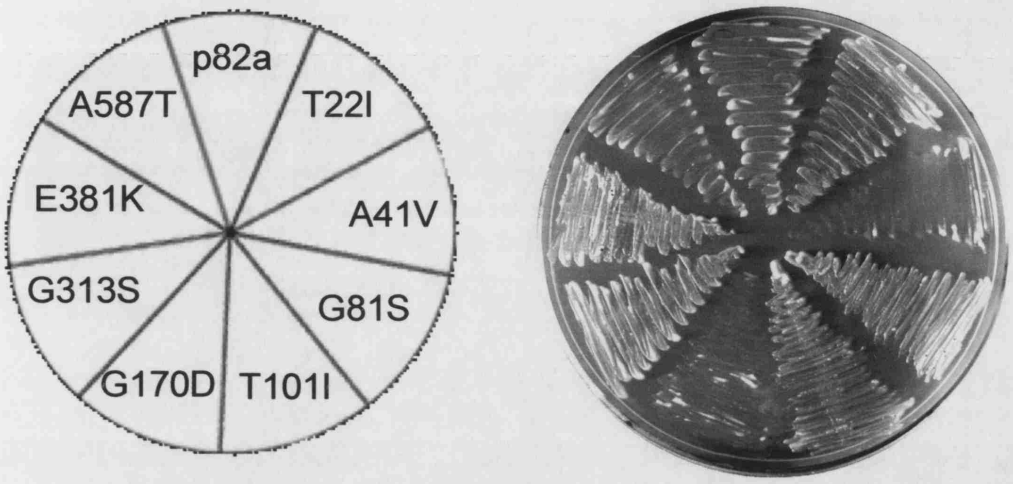


Figure 4.2)

The effect of sorbitol, an osmotic stabilizer, on the growth of the Lindquist mutants at high temperature. A plate of all 8 mutants plus the wild type (p82; w^+) was grown for 5d at 39°C on YPD containing 1.2M sorbitol. Note that only A41V*hsp82* and G170D*hsp82* are severely compromised in growth under these conditions.

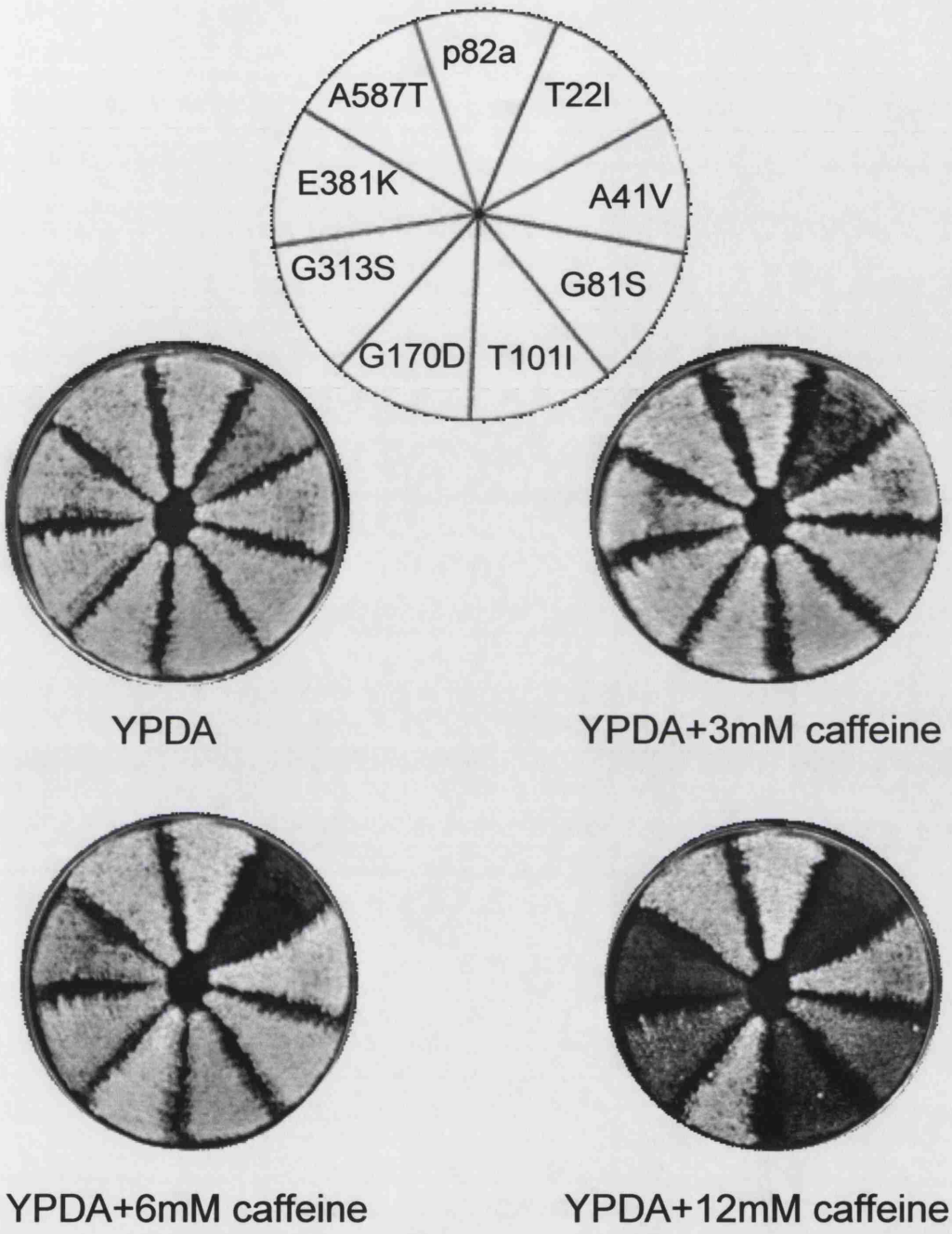


Figure 4.3)

Caffeine sensitivity of a collection of yeast mutants *ts* for growth at 37-39°C due to point mutations in Hsp82, expressed as the sole form of Hsp90 protein in these cells [Nathan, 1995]. A master plate of all 8 mutants plus the wild-type (p82; w^+) was first grown to confluency at 25°C. These cells were then replica plated onto YPD containing either zero, 3mM, 6mM, or 12mM caffeine. The plates were photographed after 5d at 20-22°C

*1.1.2 The T22I*hsp82 *mutant is defective in Rlm1p activity*

The eight *hsp82* mutants were transformed with the Rlm1p reporter plasmid (*YIL117c*-LacZ). Basal Rlm1p activity and the Rlm1p activity of cells stressed either by heat shock at 39°C for 1 hour or by the addition of 8mM caffeine for 2 hours was then determined. Although most of the mutants displayed practically normal Rlm1p activity levels, one mutant, T22Ihsp82 showed a severe lack of Rlm1p activity under all conditions (Figure 4.4). This is in contrast to the *HSF (1-583)* mutant, where basal Rlm1p activity levels are normal and there is defective Rlm1p activity under stress conditions only (Figure 3.5).

*4.2.3 T22I*hsp82 *cells show the same morphology as cell integrity pathway mutants and are sensitive to cell wall perturbing compounds*

The morphology of T22I*hsp82* cells was investigated using light microscopy under unstressed and heat shock conditions, and in the absence and presence of 1.2M sorbitol. T22I *hsp82* was enlarged at all temperatures of growth (Figure 4.5). The size of these cells was though normal in the presence of osmotic stabilisation (Figure 4.5). In contrast, in the absence of osmotic stabilisation *HSF (1-583)* cells only accumulated as enlarged, swollen cells at high temperatures (Figure 3.1b).

Work by colleagues in the lab showed that growth of the T22I*hsp82*, T101I*hsp82*, G170D*hsp82* and E381K*hsp82* mutants at room temperature in the presence of moderately-inhibitory levels of caffeine; or a shift to 39°C of *HSF(1-583)* and those *hsp82* mutants that display sorbitol-remedial ts growth (T22I*hsp82*, G81S*hsp82*, T101I*hsp82*, G313S*hsp82*, E381K*hsp82*, A587T*hsp82*; Figure 4.2) was associated with appreciable leakage of cell contents, as measured by blue colour formation with an inclusion of the phosphatase substrate 5-bromo,4-chloro,3-indoyl phosphate into the growth medium (Brown and Tuite, 1998) (not shown). Together, these observations indicate that many of the *hsp82* mutants isolated by Nathan and Lindquist (Nathan and Lindquist, 1995) are defective in the reinforcement of cell integrity needed for 37-39°C

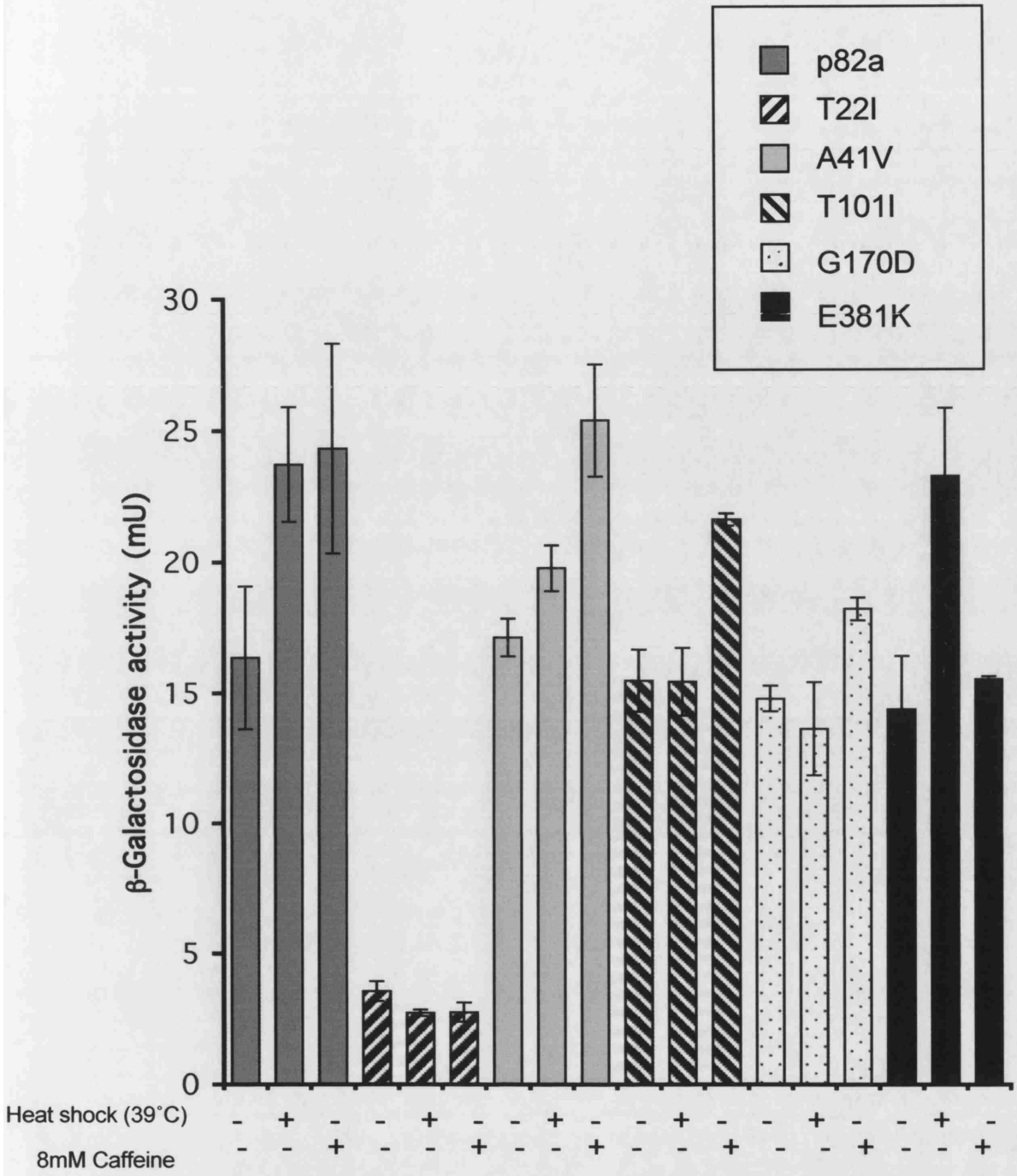


Figure 4.4)

The Rlm1p activity in five of the *hsp82* mutants from the study of Nathan & Lindquist (1995) either unstressed, subjected to heat shock (39°C for 1h) or stressed with 8mM caffeine for 1 h. Data shown is the mean and SD of three experiments.

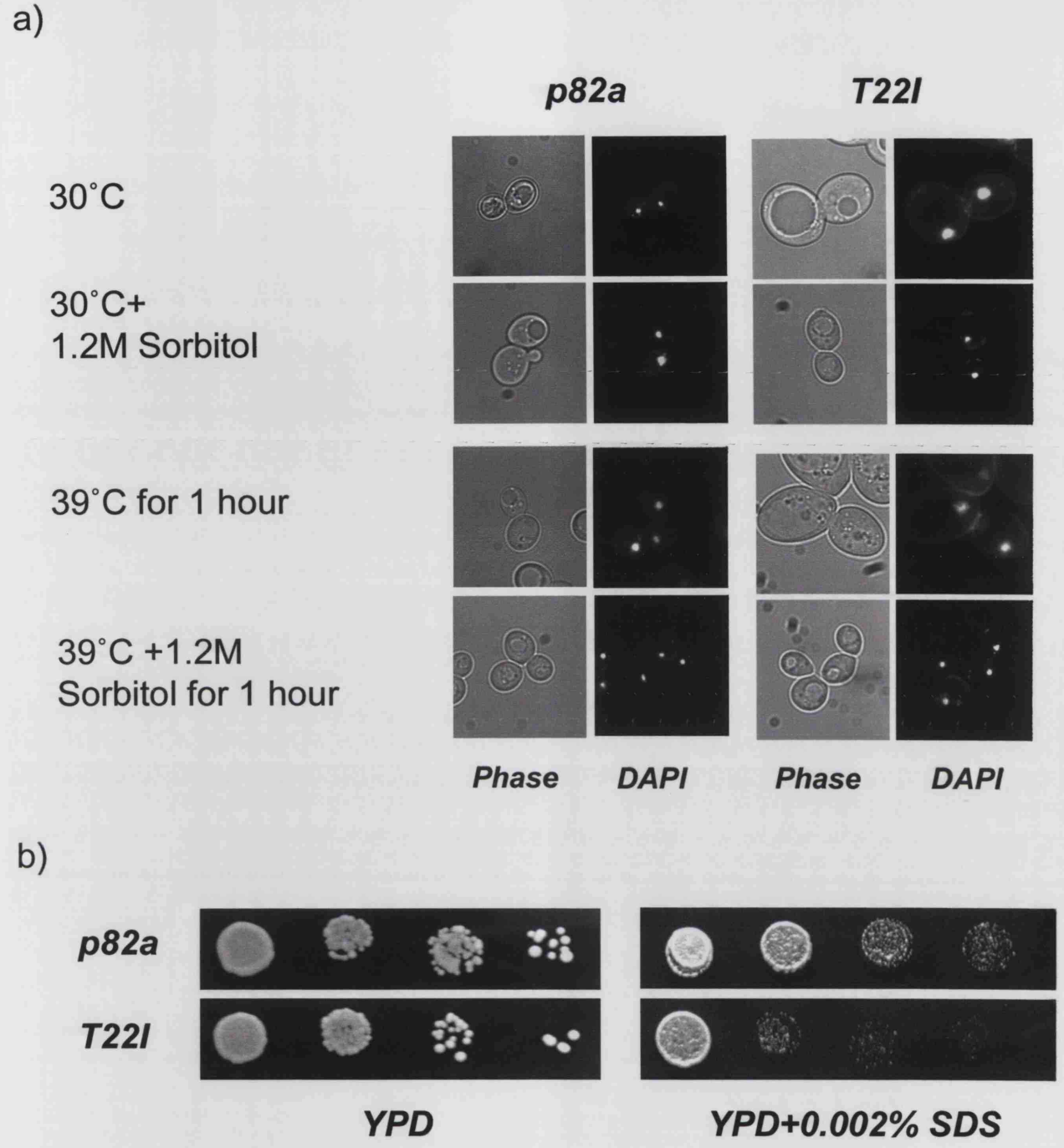


Figure 4.5)

Properties of the p82a and T22I*hsp82* strains. a) Phase-contrast and nuc lear (DAPI) stained images of unstressed and heat shocked (1h 39°C) p82a and T22I*hsp82* cells, in the absence or presence of osmotic stabilisation. Magnification x100. b) The growth of p82a and T22I cells on media containing SDS. Cells were grown to mid-log and serial diluted on either YPD agar or YPD agar containing 0.002% SDS.

growth, an effect most severe in T22I*hsp82* (Figures 4.2, 4.3, 4.4). Their caffeine-sensitive and ts phenotypes are osmoremedial, as with mutant defects in the Slt2p branch of Pkc1p-mediated signalling.

Many tests exist for cell integrity pathway mutants, including sensitivity to compounds that can potentially cause cell lysis, such as zymolyase, congo red or calcofluor white. T22I*hsp82* cells are compromised in their ability to grow in the presence of 0.002% sodium dodecyl sulphate (SDS) (Figure 4.5b), a characteristic also associated with a defect in cellular integrity.

4.2.4 In the T22Ihsp82 mutant Slt2p can still acquires dual phosphorylations in response to sensing of stress by the Slt2p MAP kinase pathway

Extracts were prepared from p82a and T22I*hsp82* cells (Table 2.1), either in growth at 25°C (unstressed), treated with 8mM caffeine for 2h at 25°C, or heat shocked from 25°C to 39°C for 1h. Levels of both total Slt2p and phosphorylated Slt2p in these extracts were then analysed (Section 2.7). As with the *HSF(1-583)* mutant, no loss of stress-induced Slt2p phosphorylation was seen in the T22I*hsp82* mutant (Figure 4.6).

4.2.5 The Hsp90-dependence of cell integrity signalling is epistatic to Mkk1p of the cell integrity MAP kinase cascade

Studies of cell integrity pathway signalling often use overactive alleles of components of the pathway to define whereabouts a particular mutation is acting. These overactive alleles are generally toxic in wild type yeast, high levels of stress activated MAP kinase activity being detrimental for the yeast cell. They are therefore often studied with the use of inducible expression systems (Hahn and Thiele, 2002; Yaakov et al., 2003). To identify where the T22I*hsp82* allele might be affecting cell integrity signalling, a constitutively active *MKK1* allele, $MKK1^{S386P}$ (Watanabe et al., 1995) was expressed in p82a (wild type) and T22I*hsp82* mutant cells. The vector used (CA-MKK1; Section 2.6.2) contained a *MET25* promoter regulated copy of this $MKK1^{S386P}$ gene. Expression of this allele was found not to suppress the *ts* phenotype of T22I*hsp82* cells at 39°C (Figure 4.7). Furthermore this expression although toxic for 30°C growth of the p82a

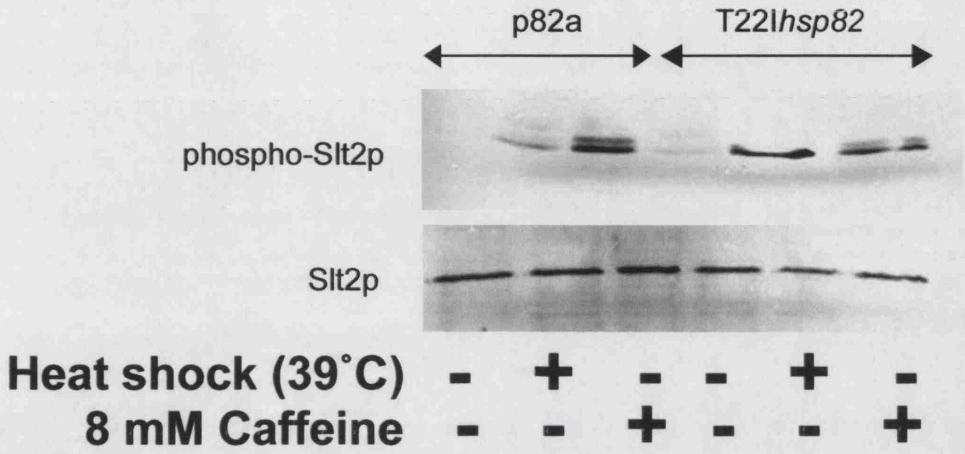


Figure 4.6)

Effects of the T22I*hsp82* mutation on Slt2p phosphorylation state in cells in growth at 25˚C (unstressed), treated with 8mM caffeine for 2h at 25˚C or heat shocked from 25˚C to 39˚C for 1h.

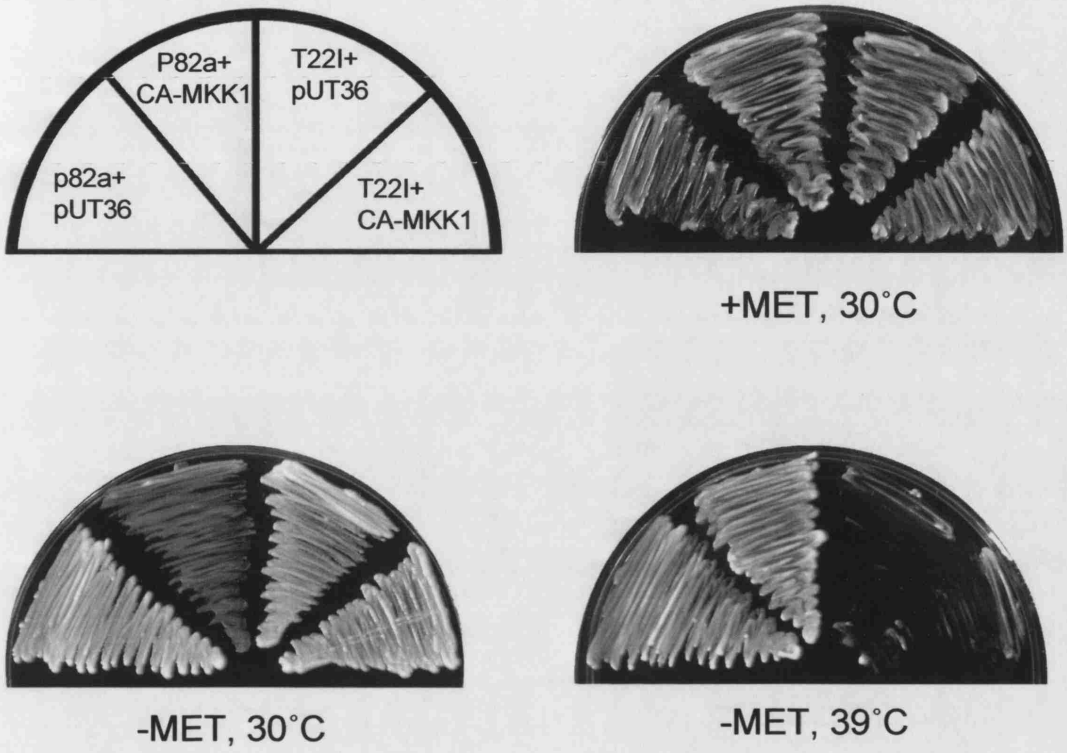


Figure 4.7)

The *MKK1*^{S386P} allele is toxic in the p82a parent of T22I*hsp82*, but not in the latter mutant. Cells containing either empty vector (pUT36) or a plasmid for a *MET25* promoter-regulated expression of an overactive allele of *MKK1* (CA-MKK1) were plated on methionine-containing or methionine-deficient medium and grown 2d at the indicated temperatures.

parent of T22I*hsp82*, was not inhibitory for growth of the latter mutant (Figure 4.7) This indicates that the T22I*hsp82* allele is acting epistatic to *MKK1* in its effects on cell integrity pathway signalling.

4.2.6 Overexpression of a constitutively active form of Rlm1p suppresses the stress-sensitive phenotype of T22Ihsp82

The above findings indicated that, as with the *HSF (1-583)* mutant (Figures 3.5, 3.9) T22I*hsp82* was causing a defect in transduction of the cell integrity activation signal from phosphorylated Slt2p to Rlm1p. The T22I*hsp82* mutant was therefore transformed with the vector for *MET25* promoter regulated (constitutively-active) AD-Rlm1p expression (Section 2.6.1). AD-Rlm1p expression led to restoration of high temperature growth at 39°C (Figure 4.8). This demonstrates that the primary defect preventing this high temperature growth of T22I*hsp82* mutant cells is at the level of Rlm1p activation.

The T22I*hsp82* allele, though not preventing Slt2p phosphorylation (Figure 4.6), is preventing relay of this activatory signal to the Rlm1p transcription factor target of this Slt2p (Figure 4.4). Since the Hsp90 chaperone is essential for the activity of many protein kinases (see Introduction), the possibility was investigated of whether there is a direct an interaction between Slt2p and Hsp90 and whether this is needed for the activity of this protein kinase.

4.2.7 Two hybrid analysis of the Hsp82:Slt2p interaction

To test whether there is an interaction between the Hsp82 molecular chaperone and Slt2p MAP kinase, an N-terminal Gal4p activatory domain (AD)- Slt2p fusion (AD-Slt2p) was generated for the purpose of measuring interactions with this molecule by the yeast two hybrid system (Section 2.8). This fusion is an active form of MAP kinase (data not shown).

PJ694-a cells (Table 2.1) expressing the AD-Slt2p were mated to PJ694-α cells expressing Hsp82-BD (a functional Hsp82 fused via its C-terminus to the Gal4p DNA binding domain), from a previous study (Millson et al., 2003). Quantitative

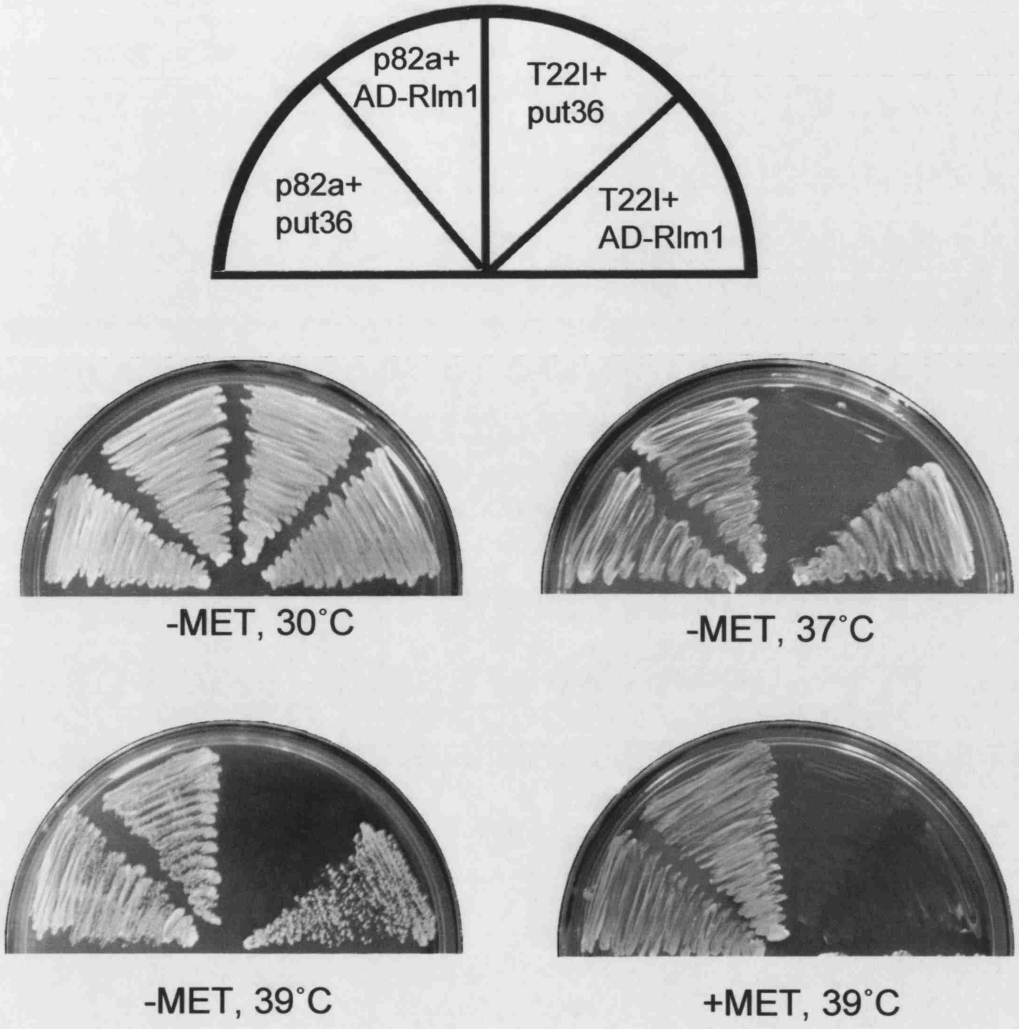


Figure 4.8)

Expression of a constitutively-active AD-Rlm1p fusion efficiently suppressed the ***ts*** phenotype of T22I*hsp82*. Cells containing either the empty vector (pUT36) or a plasmid for a *MET25* promoter regulated expression of a constitutively-active AD-Rlm1p were plated on methionine containing or methionine-deficient medium and grown 2d at the indicated temperatures.

measurement of the interaction between AD-Slt2p and Hsp82-BD in unstressed cells of the resulting diploid, also heat and caffeine stressed cells (conditions in which Slt2p would become dually phosphorylated) indicated an interaction between Slt2p and Hsp82 that increased under conditions of heat shock or caffeine stress (Figure 4.9).

4.2.8 Hsp82 interacts exclusively with the dually phosphorylated Slt2p

The significant enhancement of the two hybrid Hsp82:Slt2p MAP kinase interaction by both heat shock or caffeine stress (Figure 4.9), conditions where Slt2p becomes dually phosphorylated indicated that Hsp82 might be binding exclusively to, or more strongly to, the dually phosphorylated state of Slt2p MAP kinase. To test whether dual phosphorylation was a key requirement for the Slt2p:Hsp82 interaction, we introduced into the AD-Slt2p 'prey' fusion conservative substitutions of the two residues that must be phosphorylated for MAP kinase activity (T190A, Y192F) (see methods). We also investigated the effect of K54R, a conservative mutation that should produce a phosphorylatable yet catalytically dead version of Slt2p MAP kinase (Martin et al., 1993).

From the yeast two-hybrid analysis, it was clear that there was a negligible interaction between Hsp82 and the non-phosphorylatable (T190A, Y192F) Slt2p MAP kinase, the *GAL7* promoter directed *LacZ* expression being essentially the basal activity generated by the AD-Slt2p fusion (Figure 4.9). The Slt2p made catalytically inactive (AD-K54RSlt2p) showed slightly stronger stress-induced interaction with Hsp82-BD, compared to the wild type AD-Slt2p. This may be due to the Slt2p kinase auto-regulating its own dephosphorylation (Figure 4.9).

4.2.9 Verification of the Slt2p:Hsp82 interaction via Immobilized Metal Affinity (IMAC) Purification

To corroborate the two-hybrid data (Figure 4.9), it was decided to investigate the above interactions by protein binding, expressing in the cells an Slt2p with a C-terminal 6xhistidine tag (Slt2p-6xHis), then stressing the cells under the appropriate conditions

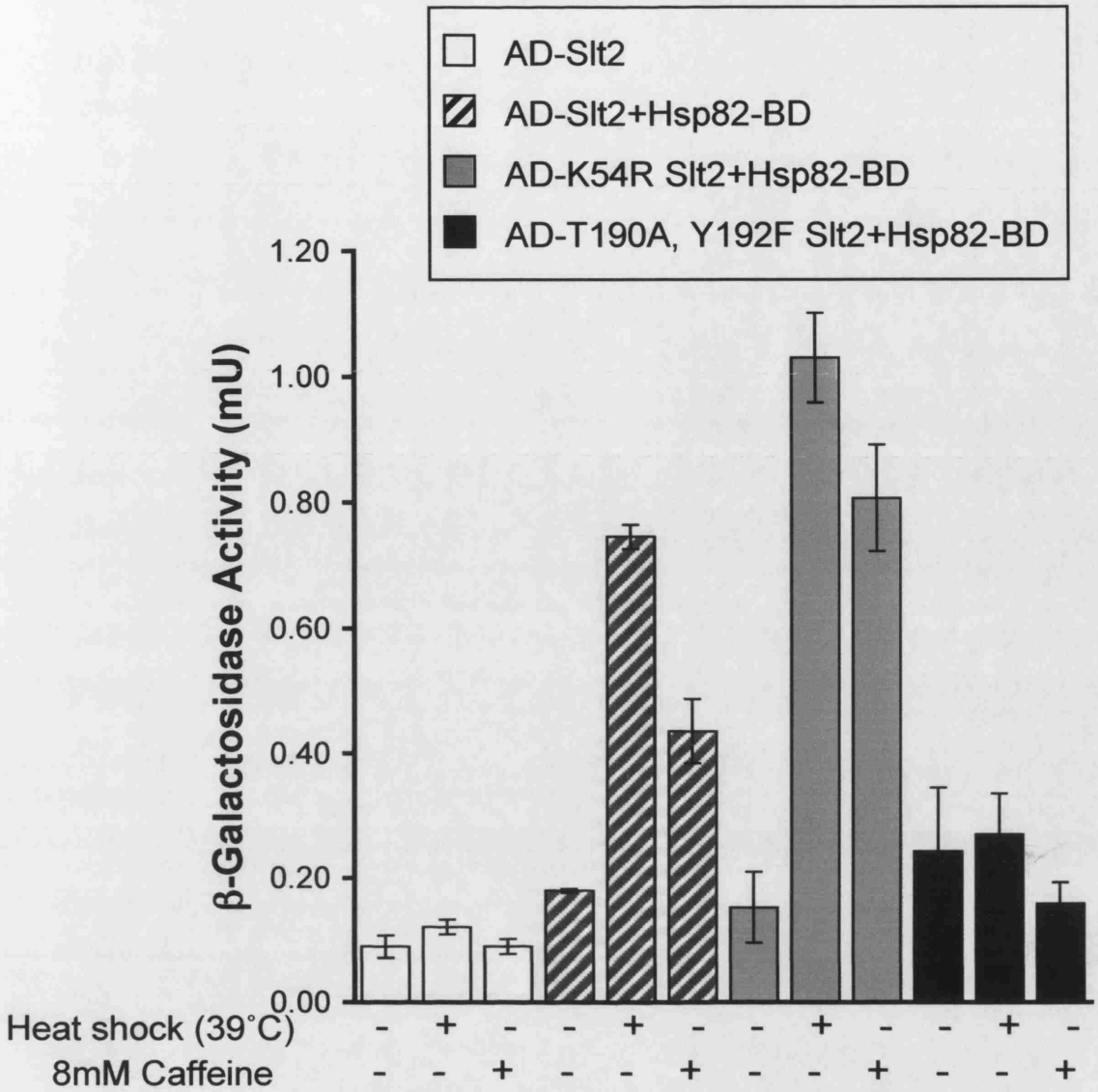


Figure 4.9)

Interaction between Hsp82 and Slt2p is reinforced under stress conditions that activate the Slt2p MAP kinase branch of Pkc1p-mediated signalling, but abolished by mutations that prevent the dual Thr^{190}/Tyr^{192}-phosphorylation of Slt2p. Shown are the basal and stress-induced two hybrid interaction of Hsp82-BD with a functional AD-Slt2p fusion; also with nonphosphorylatable (T190A,Y192F mutant) and phosphorylatable, yet catalytically dead (K54R mutant) versions of this AD-Slt2p. The control cells contained the vector for AD-Slt2p expression and empty pBDC (Millson 2003). Conditions of stress were as in Figures 4.4 and 4.6, the data being the mean and SD of three individual experiments.

and isolating the Slt2p:Hsp82 complex using nickel-nitrilotriacetate chromatography affinity columns (His-select columns, Sigma).

Vectors for a *MET25* promoter controlled expression of the Slt2p-6xHis also non-phosphorylatable and kinase-dead versions of this Slt2p-6xHis ((T190A, Y192F)-Slt2p-6xHis and K54R-Slt2p-6xHis) were constructed (Section 2.6.4). To eliminate the possibility of heterodimers formation with a native, chromosomally-encoded Slt2p (MAP kinases can undergo dimerisation in response to the structural changes induced by dual Thr, Tyr phosphorylation (Kholachev 1998)), it was decided to express these plasmids in a *slt2*Δ background. The *SLT2* gene was deleted in the p82a strain (Table 2.1) that only expresses Hsp82 thereby generating strain p82a *slt2*Δ (Table 2.1) and the Slt2p-6xHis expressing vectors were transformed into this strain. Since p82a expresses Hsp82 at constant levels under different stress conditions (Nathan and Lindquist, 1995), this meant the strength of interaction between Hsp82 and Slt2p could be accurately measured.

4.2.10 The Slt2p:Hsp82 interaction can be seen using IMAC

P82a *slt2*Δ transformants expressing Slt2p-6xHis, (T190A, Y192F) Slt2p-6xHis and K54R-Slt2p-6xHis were grown to mid log in media lacking methionine and stressed with either 8mM caffeine for 1 hour or 37°C for 1 hour. Retention of Slt2p-6xHis via His-select columns revealed that stress increased the amount of Hsp82 bound to 6xHis (Figure 4.10) confirming the yeast two-hybrid results in Figure 4.9. Hsp82 binding was abolished by removing the ability of Slt2p to acquire activatory dual phosphorylations, but not by removal of its enzymatic function (Figure 4.10).

The inability of Hsp82 to interact with the non-phosphorylatable Slt2p is important in that it clarifies the previous result of this interaction being reinforced under stress as compared to non-stress conditions (Figure 4.10). The interaction is selective for the dually phosphorylated form of Slt2p, revealing that the reason the interaction is strengthened under stress conditions is because of the shift of much of the Slt2p from an inactive to an activated, dually phosphorylated form. This also probably explains why

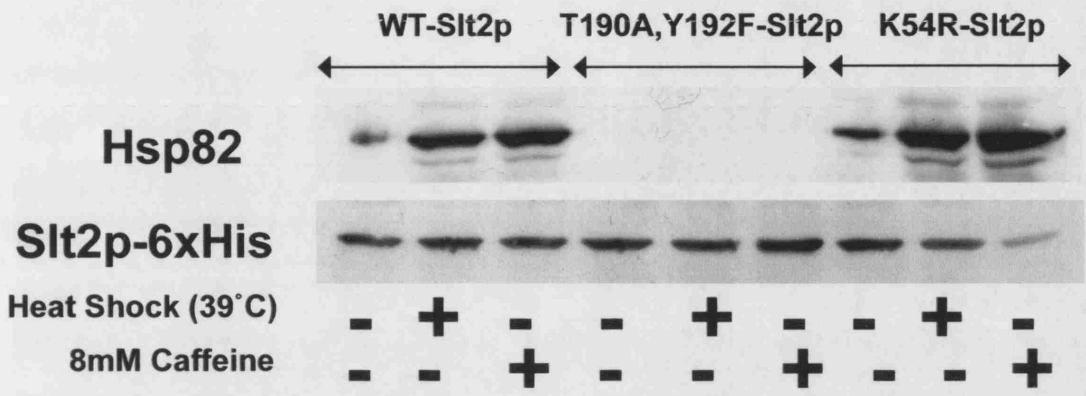


Figure 4.10)

Interaction between Hsp82 and Slt2p is reinforced under stress conditions that activate the Slt2p MAP kinase branch of Pkc1p-mediated signalling, but abolished by mutations that prevent the dual Thr^{190}/Tyr^{192}-phosphorylation of Slt2p. Basal and stress-induced interaction of Hsp82 with Slt2p-6xHis; also with nonphosphorylatable (T190A,Y192F mutant) and phosphorylatable, yet catalytically dead (K54R mutant) versions of this Slt2p-6xHis fusion in extracts from cells expressing only these forms of Hsp90 and Slt2p. Conditions of stress were as in Figures 4.4 and 4.6.

there is some basal interaction under unstressed conditions, since the cell is never truly unstressed, and a fraction of the Slt2p will be phosphorylated in growing cultures under all conditions.

4.2.11 The binding and hydrolysis of ATP by Hsp90 has direct implications for the Hsp90:Slt2p interaction

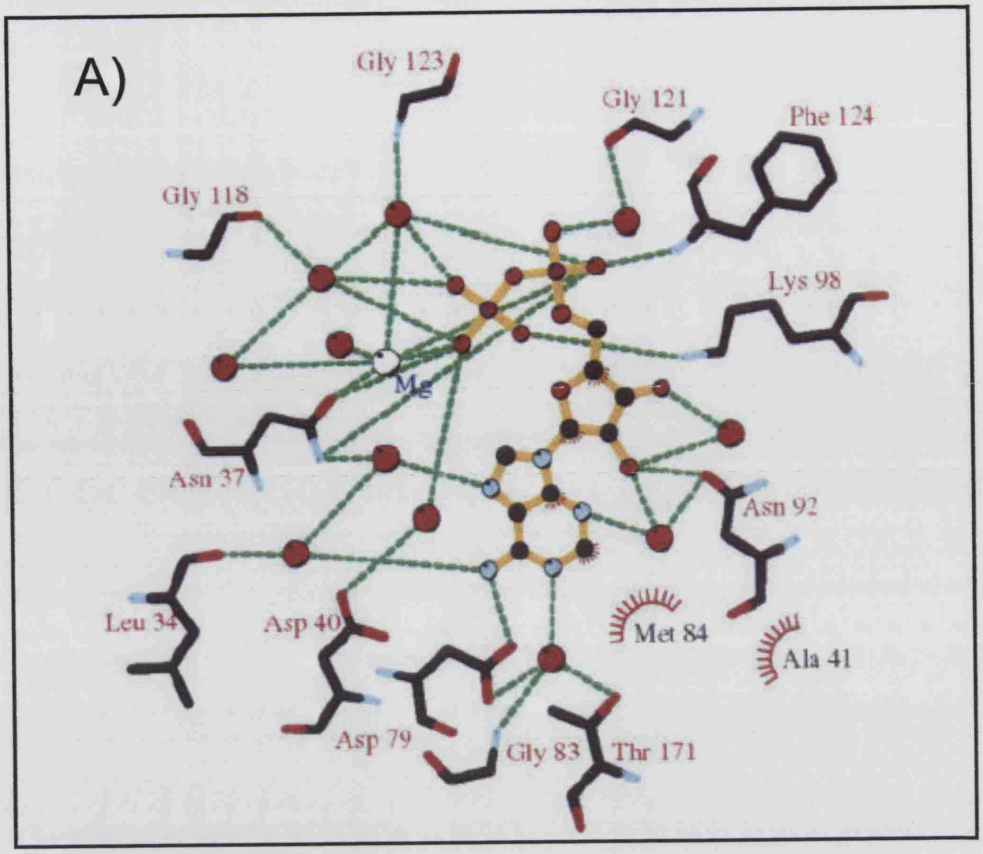
Hsp90 works as a molecular clamp, one that is dependent on the binding and hydrolysis of ATP (Panaretou et al., 1998). To check whether this binding and hydrolysis had any direct relevance on the binding of the Slt2p MAP kinase, the effects of two previously characterised point mutations in Hsp90 on the strength of two-hybrid interaction against the AD-Slt2p fusion were determined. The first point mutation, Glu33Ala (E33A) was originally studied because Glu33 of Hsp90 aligns with Glu42 of gyrase B. Glu42 functions as a general base in the ATPase mechanism of DNA gyrase B. The mutation to alanine in Hsp82 massively reduces ATPase activity, but does not prevent nucleotide binding (Panaretou et al., 1998). The second mutation, Asp79Asn (D79N) was identified from the crystal structure of the N-terminus of Hsp82 as a key residue in adenine nucleotide binding (Panaretou et al., 1998). The hydrogen bonding environment of this residue is such that even a subtle mutation to asparagine would disfavour ATP binding by generating a strongly repulsive interaction with the adenine base, with minimal disruption of the nucleotide –binding site (Figure 4.11). To summarise, E33A l allows the binding of ATP, but results in an extremely slow hydrolysis of ATP to ADP; while D79N abolishes ATP binding.

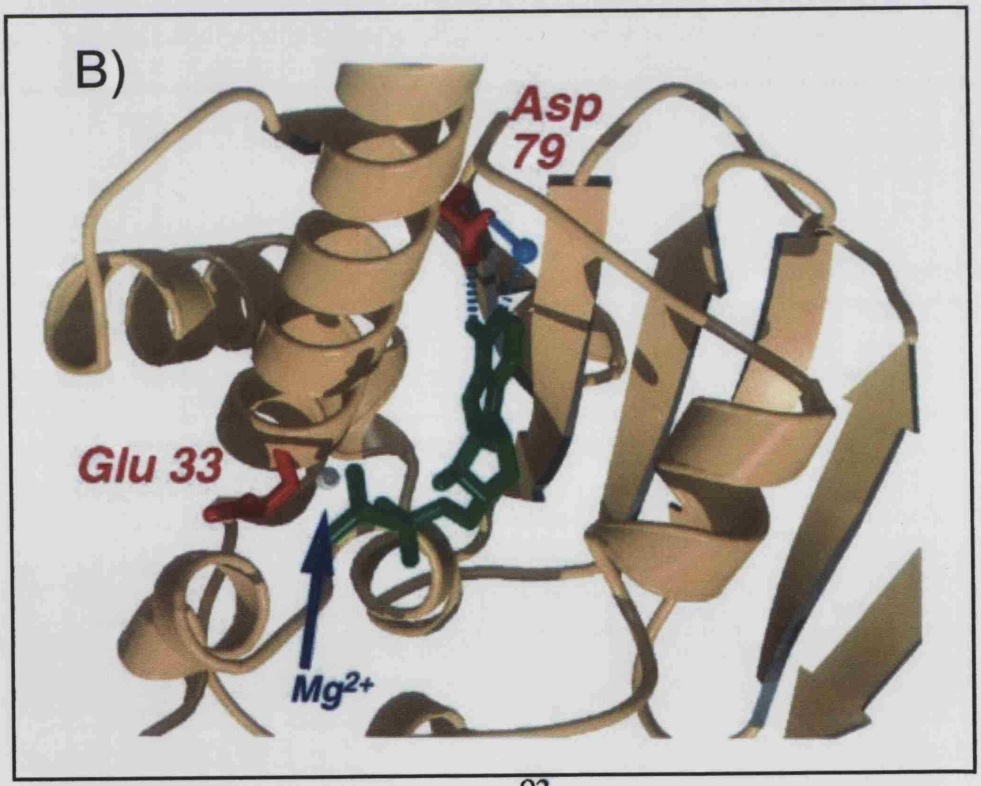
Hsp82-BD mutant constructs were made by amplifying the relevant mutant *hsp82* alleles (E33A or D79N) from plasmids used in an earlier study (Panaretou et al., 1998) using the first and second round PCR used in constructing the wild type Hsp82-BD (Millson et al., 2003). Homologous recombination within the PJ694-α yeast between these mutant PCR products and pBDC generated strains that express E33A and D79N mutant Hsp82-BD fusions, which were then mated to PJ694-a cells expressing AD-Slt2p.

Figure 4.11)

A) Residues implicated in ATP binding by Hsp90. Red spheres are water molecules. Note that only Asp79 directly contacts the bound ATP (Reproduced from Prodromou 1997).

B) A close-up of the Hsp90 N-terminal domain showing the location of Asp79 and Glu33 (in red) in the Hsp90 nucleotide binding site. The bound water forming part of the Asp79–adenine interaction is shown as a blue sphere, and the Mg21 ion which is essential for nucleotide binding is shown as a white sphere. (Reproduced from Panaretou 1998).


A)
Gly 123
Gly 121
Phe 124
Gly 118
Lys 98
Mg
Asn 37
Asn 92
Leu 34
Asp 40
Met 84
Ala 41
Asp 79
Gly 83
Thr 171


B)
Asp
79
Glu 33
Mg2+

The two-hybrid interaction of AD-Slt2p to Hsp82(E33A)-BD was increased 25-fold in unstressed conditions as compared to its interaction with the wild type Hsp82-BD (Figure 4.12). AD-Slt2p:Hsp82-BD interaction in heat-shocked and caffeine treated cells was also increased approximately 5-fold by the E33A mutation (Figure 4.12). In contrast, there was no appreciable interaction between AD-Slt2p and Hsp82-BD when the D79N mutation was introduced in the Hsp82-BD fusion. The E33A mutation will allow the binding of ATP, but a very slow rate of ATP hydrolysis. This is effectively 'locking' the Hsp82 into the late stage chaperone complex, a form that can also be induced, at least *in vitro* by the addition of molybdate to the Hsp82 system (Hartson et al., 1999; Pratt and Toft, 1997). This late stage form may be binding a select group of co-chaperones such as Sba1p and Cdc37p, and it appears that the dually phosphorylated Slt2p is bound to this ATP bound form of Hsp82 molecular chaperone. Any trace interaction to the D79N mutant Hsp82-BD fusion (Figure 4.12) might be explained by the basal activity of the AD-Slt2p and the presence of wild-type Hsp82 in the yeast two-hybrid strains (it is necessary to keep the wild type Hsp90 in the cells used for these experiments as the E33A and D79N mutant forms of Hsp82 do not provide the essential Hsp82 function (Panaretou et al., 1998)).

A *TRP1* vector for *GPD1* promoter-regulated expression of a C-terminally 6xHis-tagged Hsp82 (Hsp82-6xHis); also E33A or D79N mutant forms of this Hsp82-6xHis was obtained from Dr. Stefan Millson. PJ694 cells were transformed with these constructs; the 6xHis-tagged protein of unstressed, heat-shocked and caffeine stressed cells of the resulting transformants then being isolated on His-select columns and analysed by western blotting. Probing of the blots with anti-Slt2p antisera confirmed that the association of Hsp82-6xHis with Slt2p was increased by both stresses and the presence of the E33A mutation in the Hsp82-6xHis, but abolished by D79N (Figure 4.13).

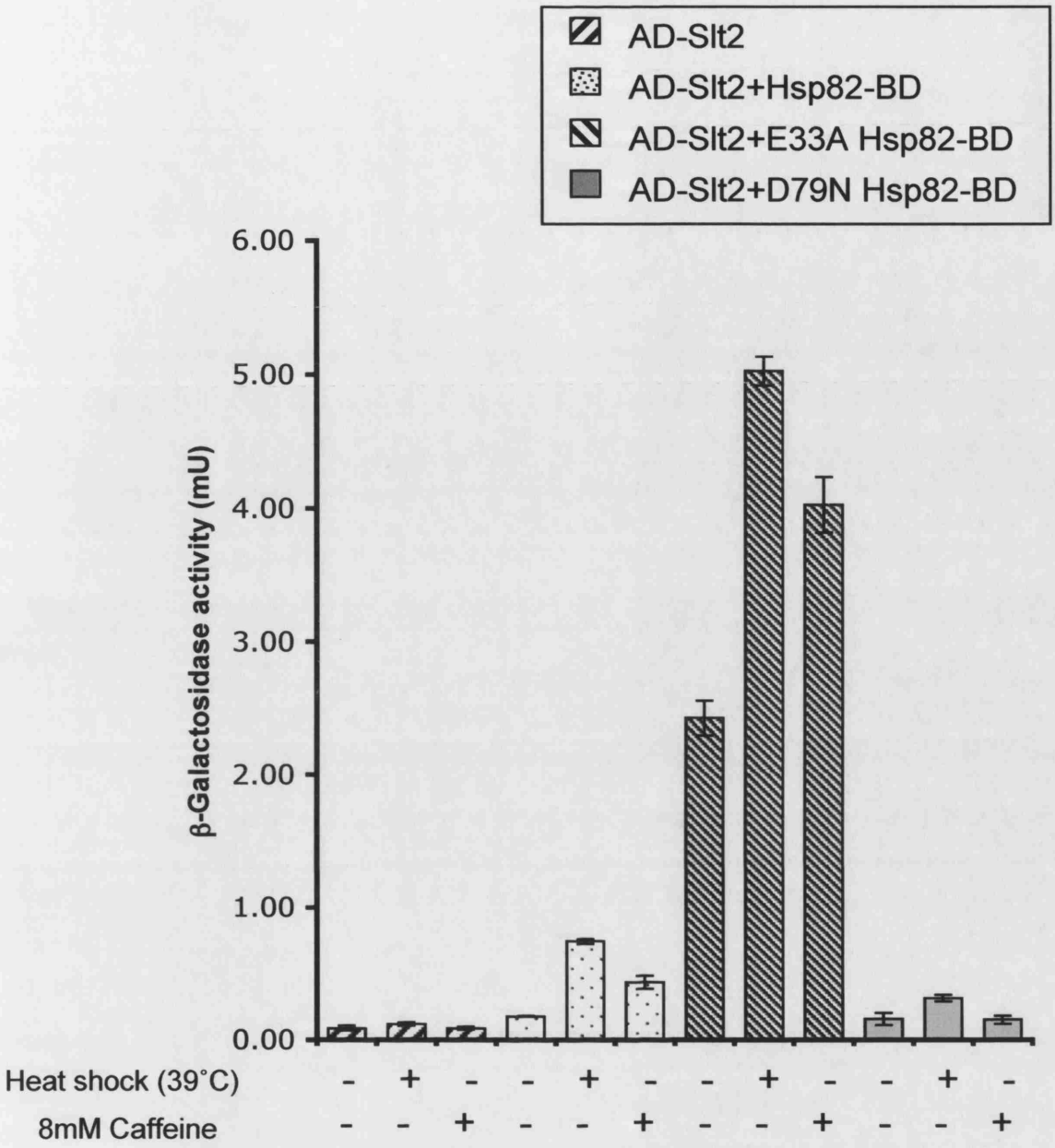


Figure 4.12)

The ATP binding and hydrolysis capabilities of Hsp82-BD affect its interaction with AD-Slt2p in the yeast two-hybrid system. Cells were grown to mid-log and then stressed under the same conditions as in figures 4.4, 4.6. The strength of interaction between Slt2p and Hsp82p was measured by β-Galactosidase activity. Data shown is the mean and SD of three separate experiments.

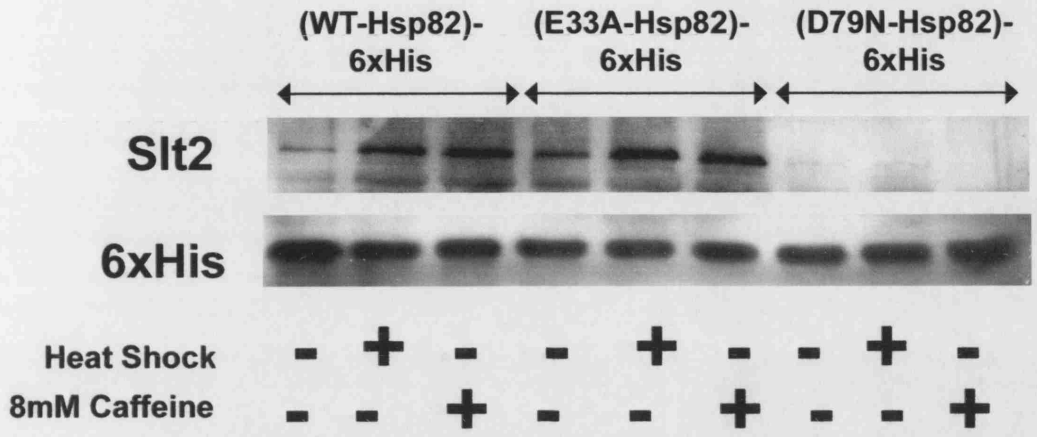


Figure 4.13)

The ATP binding and hydrolysis capabilites of Hsp82p affect its strength of binding to Slt2p. Hsp82(WT/E33A/D79N)-6xHis was isolated as previously described from cells stressed under the conditions in Figures 4.4, 4.6. Western blots were probed with either 6xHis-antisera or Slt2p antisera.

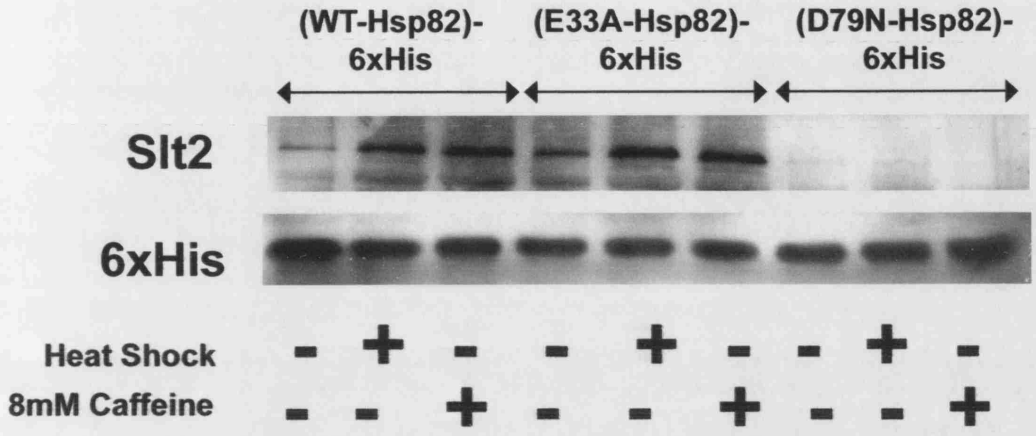


Figure 4.13)

The ATP binding and hydrolysis capabilites of Hsp82p affect its strength of binding to Slt2p. Hsp82(WT/E33A/D79N)-6xHis was isolated as previously described from cells stressed under the conditions in Figures 4.4, 4.6. Western blots were probed with either 6xHis-antisera or Slt2p antisera.

4.2.12 The Slt2p MAP kinase interacts more strongly with Hsp82, compared to the 97% similar Hsc82 isoform of Hsp90

As mentioned previously, *S. cerevisiae* expresses two almost identical isoforms of Hsp90, Hsc82p (constitutively expressed) and Hsp82 (expressed under heat shock conditions). Because of the 97% sequence similarity of the two proteins, it has always been assumed that they have identical functions and client protein specificity. Nevertheless, it appears Hsp82 overexpression but not Hsc82 overexpression can suppress the *HSF(1-583)* phenotype (Morano et al., 1999), the Hsp82 being required to allow dually-phosphorylated Slt2p to activate Rlm1p (Chapter 3). We decided to measure the relative strengths of Slt2p interaction with Hsc82p and with Hsp82 using Hsp82-BD and Hsp82-BD two hybrid constructs created in an earlier study (Millson et al., 2003).

Remarkably, heat and caffeine stresses generated smaller increases in the two-hybrid association of the AD-Slt2p fusion with Hsc82-BD as compared to the almost identical Hsp82-BD "bait" (Figure 4.14). It appears that this discrimination between the Hsp90 isoforms is not absolute. Rather, Hsc82p exhibits a weaker binding to Slt2p compared to Hsp82.

To obtain direct protein binding data of whether this association is affected by the isoform of yeast Hsp90 we made use of strains expressing just one of these isoforms of Hsp90. PP30(pHSC82) and PP30(pHSP82) (Table 2.1) express, to similar level, from the constitutively-active *HSC82* promoter, *just* Hsc82p *or* Hsp82 respectively (Figure 4.15). These two strains were transformed with a vector for the expression of Slt2p-6xHis (Section 2.6.4). Retention of 6xHis-tagged Slt2p on His-select columns, revealed an appreciable, stress-reinforced association of the Slt2p-6xHis with the Hsp82 in the extracts from PP30(pHSP82) (Figure 4.16). However in the same experiment the Hsc82 in the extracts from the identically-treated PP30(pHSC82) cells interacted considerably more weakly with Slt2p-6xHis (Figure 4.16).

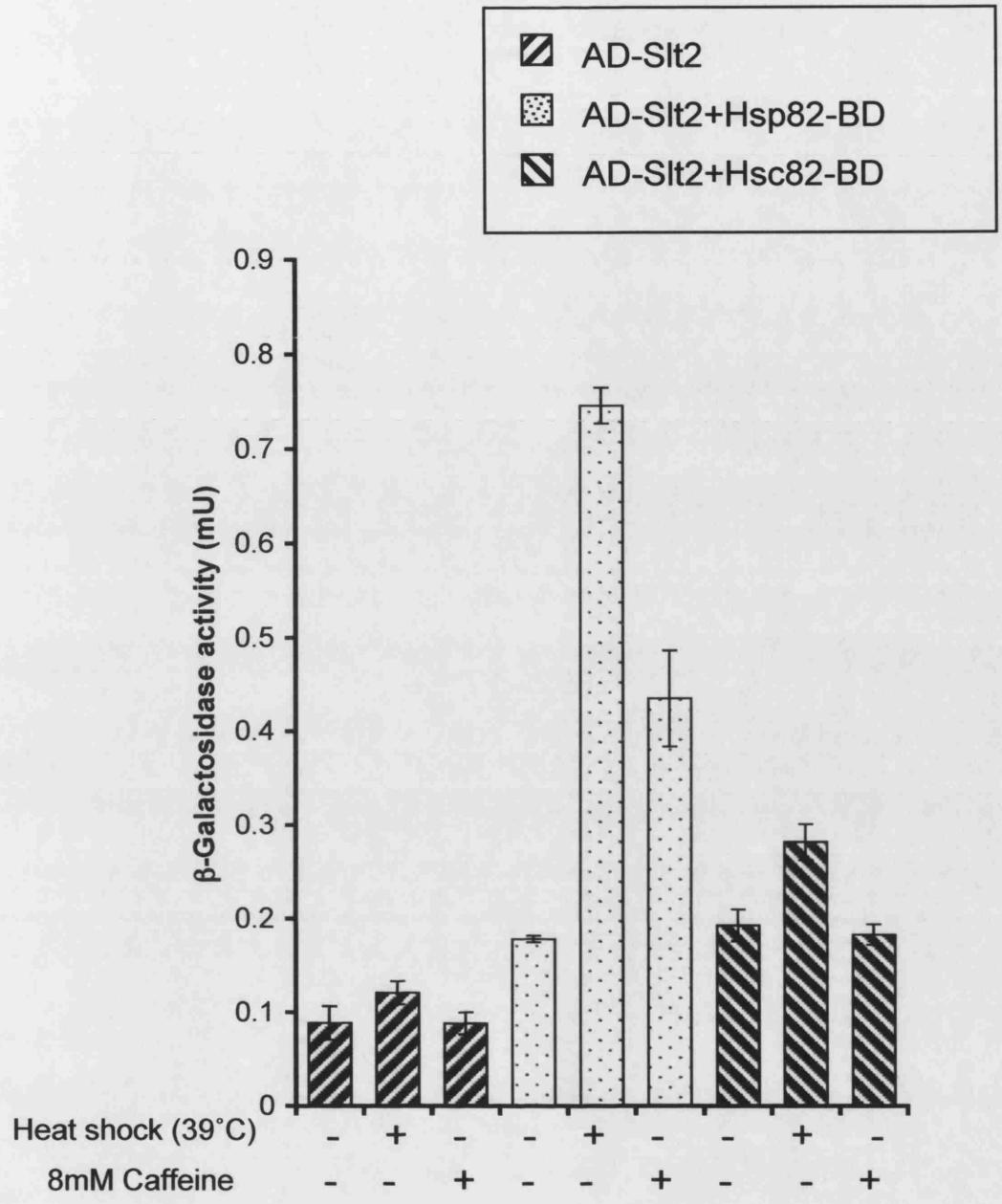


Figure 4.14)

The Slt2p MAP kinase preferentially binds to the Hsp82 isoform as compared to the 97% identical Hsc82 isoform of Hsp90 as shown by yeast two-hybrid system. As previously, cells were grown to mid-log and stressed with the appropriate conditions. The interaction was measured via β-Galactosidase assay and the mean and SD of three separate expreiments are shown.

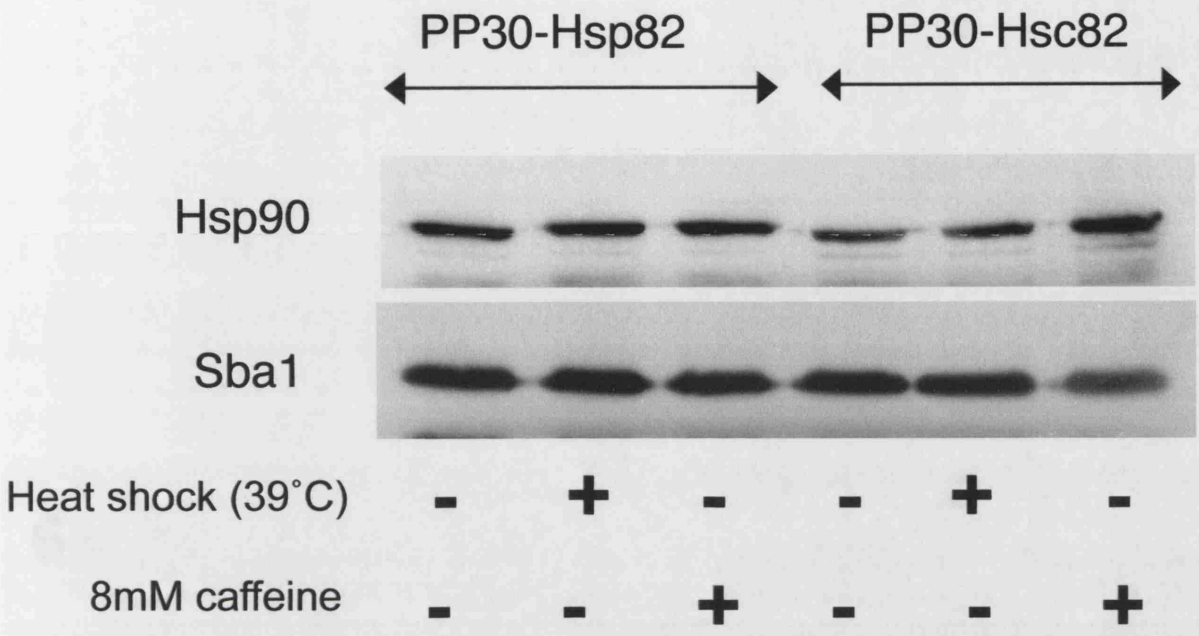


Figure 4.15)

Levels of Hsp90 in pp30 strains (expressing either Hsp82p or Hsc82p) are constant under stress conditions. These were grown to mid-log phase and were either left unstressed, or stressed 1h by heat-shocked at 39°C or 8mM caffeine at 25°C whereupon the total cell protein was extracted, 10μg of which was run on a 7.5% SDS-polyacrylamide gel. The Hsp90 levels were measured with an antibody that recognises the C-terminus of both Hsp90 isoforms, and Sba1p was used as a loading control.

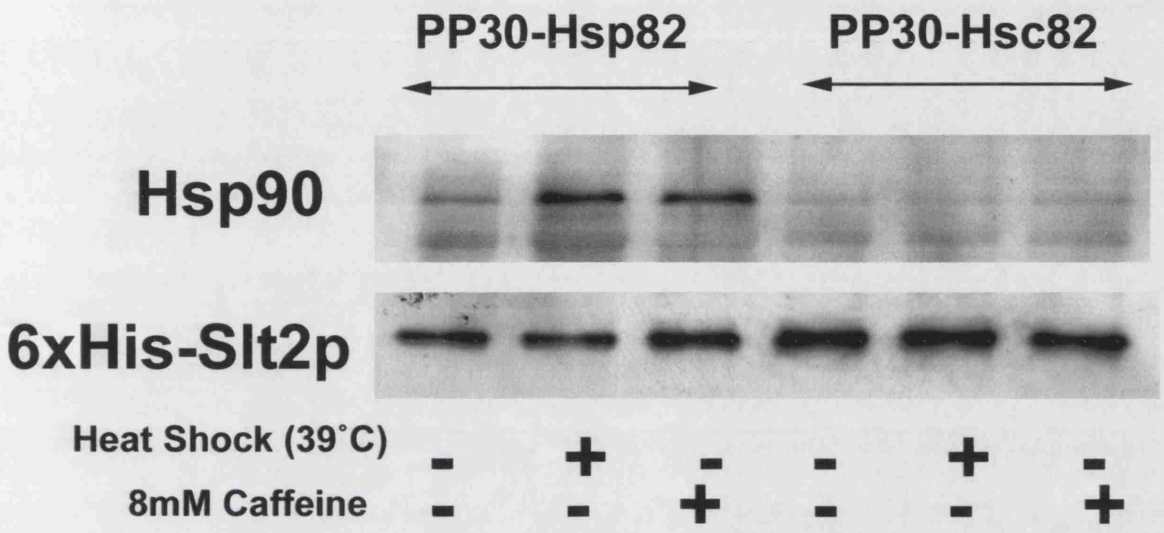


Figure 4.16)

Measurement of Hsp90 retained on talon resin in cells of the PP30(pHSC82) and PP30(pHSP82) strains expressing Slt2p-6xHis. Caffeine and hea t stress reinforce the Slt2p-Hsp82p interaction to a greater extent than the interaction of this same Slt2p-6xHis with the almost identical Hsc82p. Conditions of stress were as in Figure 4.15.

4.2.13 Hsp82 interacts with the N-terminal MAP kinase domain of Slt2p

Slt2p consists of two main sections 1) an N-terminal MAP kinase domain that is well conserved throughout eukaryotic organisms, that is capable of being dually phosphorylated, and that contains the site for catalytic activity and 2) a C-terminal section which has no homology with any known protein, contains a long polyglutamine tract and is partially dispensable for function (Soler et al., 1995). To see whether Hsp90 binds to the MAP kinase domain, a truncated AD-Slt2p fusion which contained only residues 1-328 of Slt2p was created (the N-terminal MAP kinase domain) by creating a first round Y2H reverse primer (*SLT21-328Y2HR*) which annealed to bases 961-984 of *SLT2*. The truncated *SLT2* PCR product was used as a template in the 2nd round of PCR. Interestingly, Hsp82-BD interaction was retained using just the N-terminal MAP kinase section of Slt2p (residues 1-328) in the yeast two hybrid system (Figure 4.17). Again interaction was stronger with Hsp82 as compared to the Hsc82 isoform of yeast Hsp90 (Figure 4.17). The fact that some MAP kinases of yeast (Slt2p) bind Hsp82 but whereas others do not (Fus3p from work carried out in our lab) suggests that subtle differences in sequence/structure determine whether the MAP kinase domain binds Hsp82.

4.2.14 The Slt2p:Hsp82 interaction can be strengthened with Molybdate

In vitro studies have shown that it is possible to *stabilise* Hsp90:client complexes with molybdate (Soti et al., 1998). The MoO_4^{2+} oxyanion is thought to inhibit the final ATPase step of the Hsp90 chaperone cycle and associated release of the activated client protein by operating as a transition state analogue of phosphate (Hartson et al., 1999; Pratt and Toft, 1997; Young and Hartl, 2000). Initially we investigated the effects of incorporating molybdate into the growth medium used in two hybrid screening, but found that the high molybdate concentrations needed to obtain any effects (20-40mM) were strongly inhibitory to growth of the yeast in media required for Y2H (not shown). Instead, p82a *slt2*Δ cells containing the plasmid for Slt2p-6xHis expression were grown to mid log and

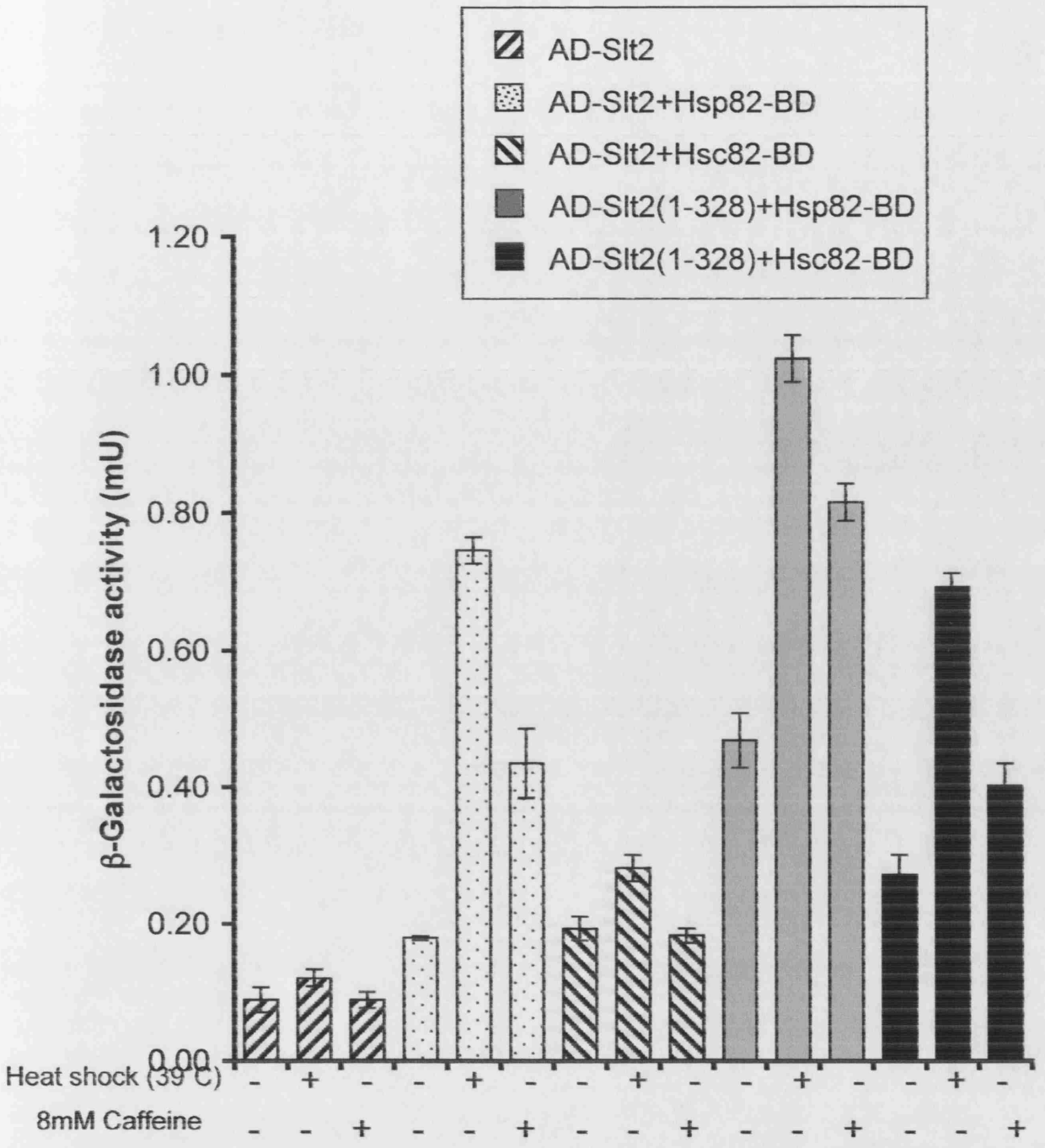


Figure 4.17)

The C-terminal section of Slt2p (residues 329-484) is dispensable for interaction with Hsp82 as shown by yeast two-hybrid. As previously, cells were grown to mid-log and stressed with the appropriate conditions. The interaction was measured via β-Galactosidase assay and the mean and SD of three separate results are shown.

exposed to 20mM and 50mM of sodium molybdate for 1 hour. IMAC retention revealed a significant enhancement of the Slt2p:Hsp82 interaction as compared to when no molybdate was present (Figure 4.18). This is a characteristic of late stage Hsp90-client complexes, MoO_4^{2+} stabilising the interactions of Hsp90 with a number of client proteins (steroid receptors, protein kinases and heat-denatured luciferase) (Hartson et al., 1999; Pratt, 1997).

4.2.15 The T22Ihsp82 mutation does not prevent Slt2p binding

Having observed that the T22I*hsp82* mutant displays a clear cell wall defect, due to an inability to activate Rlm1p-dependent gene expression (Figures 4.2-4.8), it was decided to determine whether the T22I*hsp82* mutation abolishes interaction between Slt2p and Hsp82. As mentioned previously, it had already been shown that T22I mutation caused increased ATPase activity of purified Hsp82 (Prodromou 2000) so we considered the possibility that the Slt2p:Hsp82 interaction may be weakened by this mutation. The *SLT2* gene was deleted in the T22I*hsp82* strain and this strain was subsequently transformed with the plasmid for Slt2p-6xHis expression. Isolating the Slt2p-6xHis from unstressed and stressed cells it was found that the interaction had not been lost, but if anything had actually been strengthened (Figure 4.19).

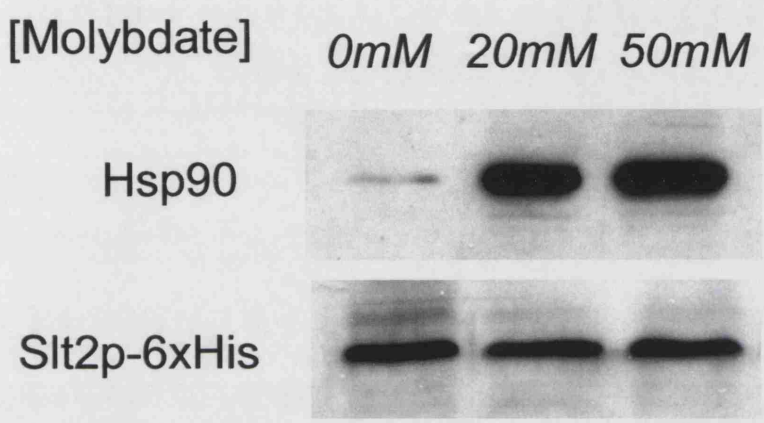


Figure 4.18)

Molybdate stabilises the Slt2p:Hsp82 interaction. Cells were grown to mid-log and then incubated with either 0, 20 or 50mM sodium molybdate for 1 hour at 25°C. Slt2p-6xHis was isolated and retained. Slt2p-Hsp82p interaction was measured using anti-Hsp90 and 6xHis antisera.

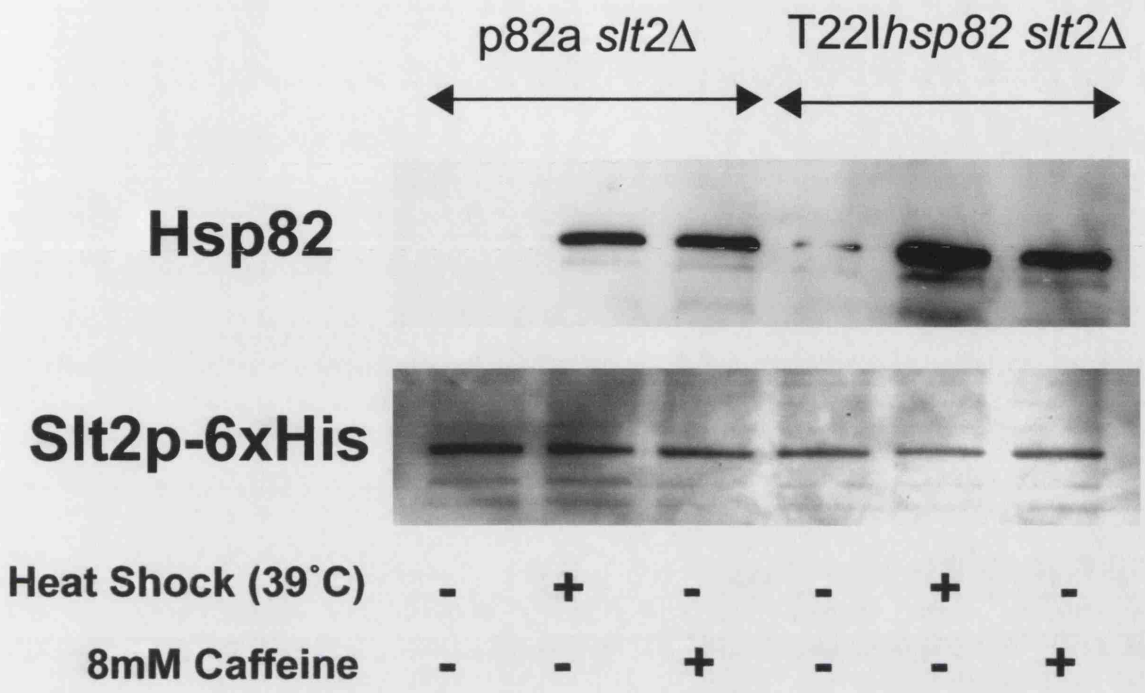


Figure 4.19)

The Slt2p:Hsp82 interaction is conserved in the T22I*hsp82* mutant. Extracts were prepared from stressed and unstressed p82a and T22I*hsp82* cells expressing Slt2p-6xHis then retention of Hsp82 was measured as in Figure 4.15.

4.3 Conclusion

Analysis of a collection of mutants containing single point mutations in Hsp82 as their sole Hsp90 revealed that one such mutant, T22I*hsp82*, displays an osmoremedial phenotype, much in the same way that *HSF(1-583)* does. Yet on closer examination, a distinction between the two mutants was apparent. The defect in *HSF(1-583)* appears only under stress conditions, presumably because there is not enough Hsp82 in the cell to allow the Slt2p MAP kinase activation of Rlm1p. The T22I*hsp82* mutant appears to display this defect at all temperatures of growth, by a mechanism as yet unexplained.

Thr22 is some way from the mouth of the nucleotide-binding pocket and is unable to make direct contact with bound nucleotide. The T22I mutation has a dramatic effect on the ATPase activity of the purified chaperone, increasing it by 5-fold compared to that of wild type chaperone at 30°C, although affinity for AMP-PNP is somewhat decreased. As the T22I mutant is fully capable of binding and hydrolysing ATP, it is not immediately obvious why this 'hyperactive' mutant is causing defective Slt2p function *in vivo*. The T22I mutant Hsp82 binds Slt2p just as well as the wild type version-it could be that the T22I protein is unable to bind other components (such as co-chaperones) to produce a fully active Slt2p molecule (see discussion). As the ATP-bound mature Hsp90 complexes are the stage in which client protein activation takes place, a substantial decrease in the lifetime of that state might impair activation of essential client proteins even under normal growth conditions. Alternatively, the Hsp82 (ATP bound form):Slt2p complex might become hydrolysed prematurely before this complex can engage in a docking system with Rlm1p and activate the latter.

This chapter describes analysis of the T22I*hsp82* cell integrity defect revealing that Slt2p is a novel (and potentially model) client protein of Hsp82, Slt2p. The compromised Hsp90 function of T22I *hsp82* cells prevents Slt2p activation of Rlm1p. This work has uncovered some of the novel aspects of the Slt2p:Hsp82 interaction, for example the

requirement for Slt2p to be dually phosphorylated (i.e. in it activated form) in order to bind Hsp82.

5 Using yeast to search for the mammalian equivalent of the Slt2p:Hsp82 interaction

5.1 Introduction

In the previous two Chapters, a function of the Hsp82 molecular chaperone in the capacity of the yeast Slt2p MAP kinase to activate its downstream target, Rlm1p. Although yeast is a good model system in which to demonstrate and examine such an interaction, one must consider the wider implications. Does a similar interaction exist in human cells and what is its biological significance? There is such a strong degree of homology between the *H. Sapiens* and *S. cerevisiae* Hsp90 chaperone proteins that it is likely that they act in similar ways. Only a few MAP kinase-related proteins, such as MAK, MRK and MOK in human tissue have been shown to bind Hsp90, but it has not been shown these interactions are important for functioning of these kinases (Miyata et al., 2001; Pratt and Toft, 1997). MAP kinases are a very well conserved group of proteins, yet it appears that despite this similarity, some will bind Hsp90 whereas others will not (Miyata and Yahara, 2000). To date, no consensus motif for MAP kinase binding to Hsp90 has been found.

Many MAP kinases such as ERK2 have been implicated as important molecules in tumorgenic growth and therefore are interesting targets for cancer therapy studies. If more Hsp90-dependant MAP kinases could be found, then drugs that reduce Hsp90 function could be used to down regulate these potentially dangerous signalling molecules in tumours.

In this Chapter, we study the potential for an equivalent Slt2p:Hsp82p interaction in mammalian cells by investigating; (1) whether Slt2p can bind the human isoforms of Hsp90, (2) whether a *H. Sapiens* functional homologue exists for the yeast Slt2p MAP kinase; and (3) whether this functional homologue can bind Hsp90.

5.2 Results

5.2.1 *Slt2p interacts with the human isoforms of Hsp90, Hsp90α and Hsp90β*

Using Gal4p DNA binding domain fusions to the human Hsp90 isoforms, Hsp90α and Hsp90β (Hsp90α-BD and Hsp90β-BD) provided by Dr. S. Millson, the yeast two-hybrid interaction with AD-Slt2p was measured (Figure 5.1). As in Chapter 4, we observed a significant interaction, one that was markedly enhanced with addition of stress in the form of heat shock (39°C for 1h). There was a stronger AD-Slt2p binding to Hsp90β (the isoform constitutively-expressed in mammalian tissues) over Hsp90α (the heat-inducible isoform), whereas with the yeast isoforms binding is stronger to the heat-inducible isoform, Hsp82 (Figures 4.14, 4.16). There also appeared to be a lack of reinforcement of the interaction following the addition of 8mM caffeine (Figure 5.1). This suggests subtle differences in the interaction of Slt2p with the yeast and the human isoforms of Hsp90.

5.2.2 *Mammalian ERK2 has a high degree of homology to yeast Slt2p MAP kinase*

The Slt2p protein sequence was searched against all proteins currently in the RCSB protein data bank (PDB). The 5 closest matches found were ERK2 complexed with different inhibitors (Figure 5.2). ERK2 is one of the best studied MAP kinase proteins, and the high-resolution structures of both the un-phosphorylated and di-phosphorylated forms of the molecule have been solved (Khokhlatchev et al., 1998). Although the additional C-terminal section of the Slt2p is not present in the ERK2 sequence, the N-terminal sequence (which contains the catalytic site and dual phosphorylation sites) is so well conserved (50% identical residues, 27% similar residues, Figure 5.2), that it was suggested that this molecule could be a good starting point for the investigation.

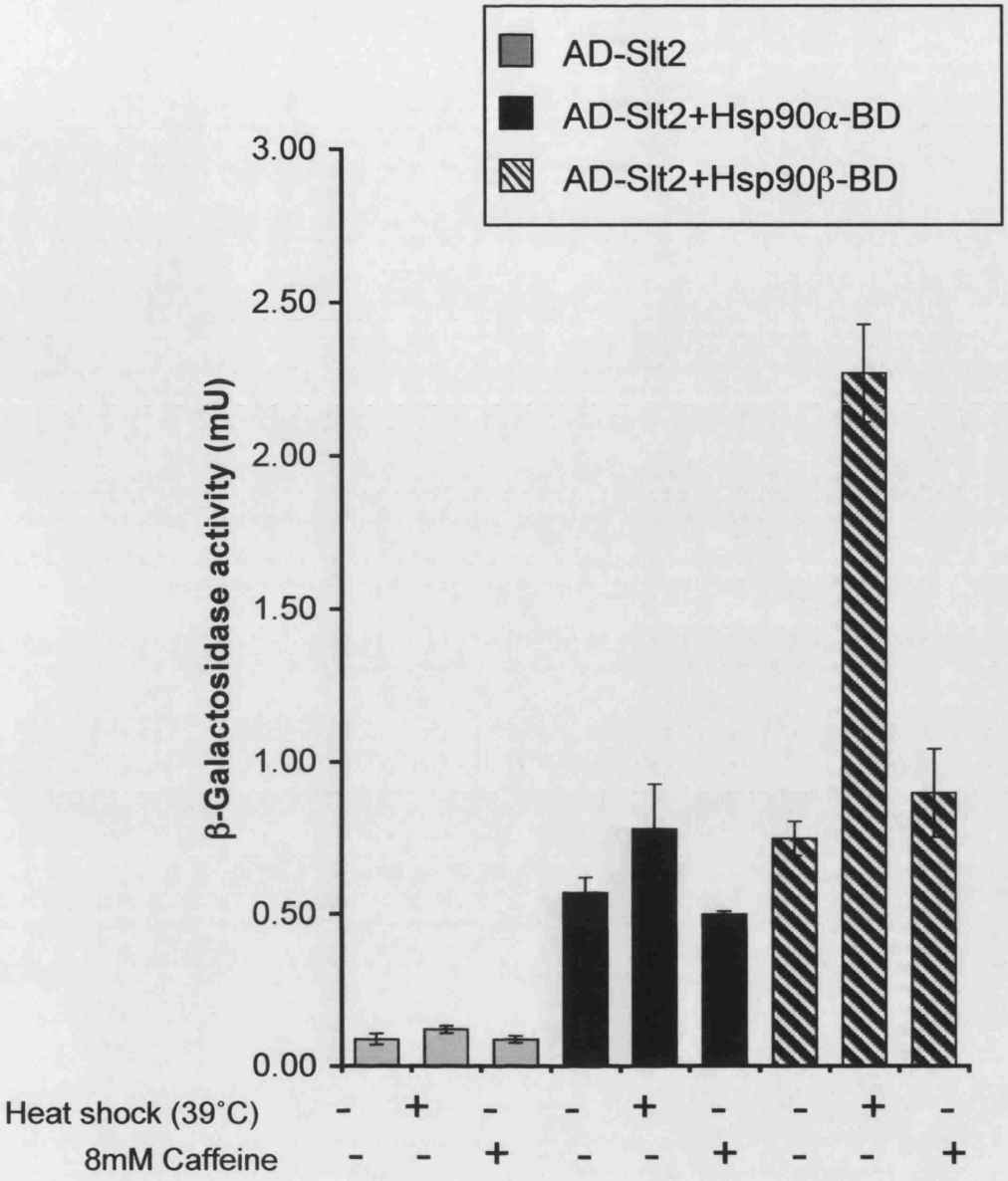


Figure 5.1)

Slt2p interacts with human Hsp90 isoforms Hsp90α and Hsp90β in the yeast two hybrid system. As previously, cells were grown to mid-log and stressed with conditions as in Figs. 4.14, 4.16. The interaction was measured via β-Galactosidase assay and is the mean and SD of three separate experiments.

A)

PDB protein structure(s) homologous to Slt2p	Protein Alignment:		
	P-Value	% Identical	% Similar
4erk The complex structure of the map kinase Erk2/olomoucine	2.90E-72	50	27
1lez (Chain A) Crystal structure of MAP kinase p38 complexed to the docking site of its activator mkk3b	1.00E-65	46	27
1jnk The c-jun n-terminal kinase (jnk3s) complexed with mg amp-pnp	2.60E-54	38	31
1ukh (Chain A) Structural basis for the selective inhibition of jnk1 by the scaffolding protein jip1 and sp600125	7.40E-52	38	30
1unl (Chains A&B) Cyclin-Dependent kinase	Chain A=6.7e-37 Chain B=6.7e-37	37 37	31 31

B)

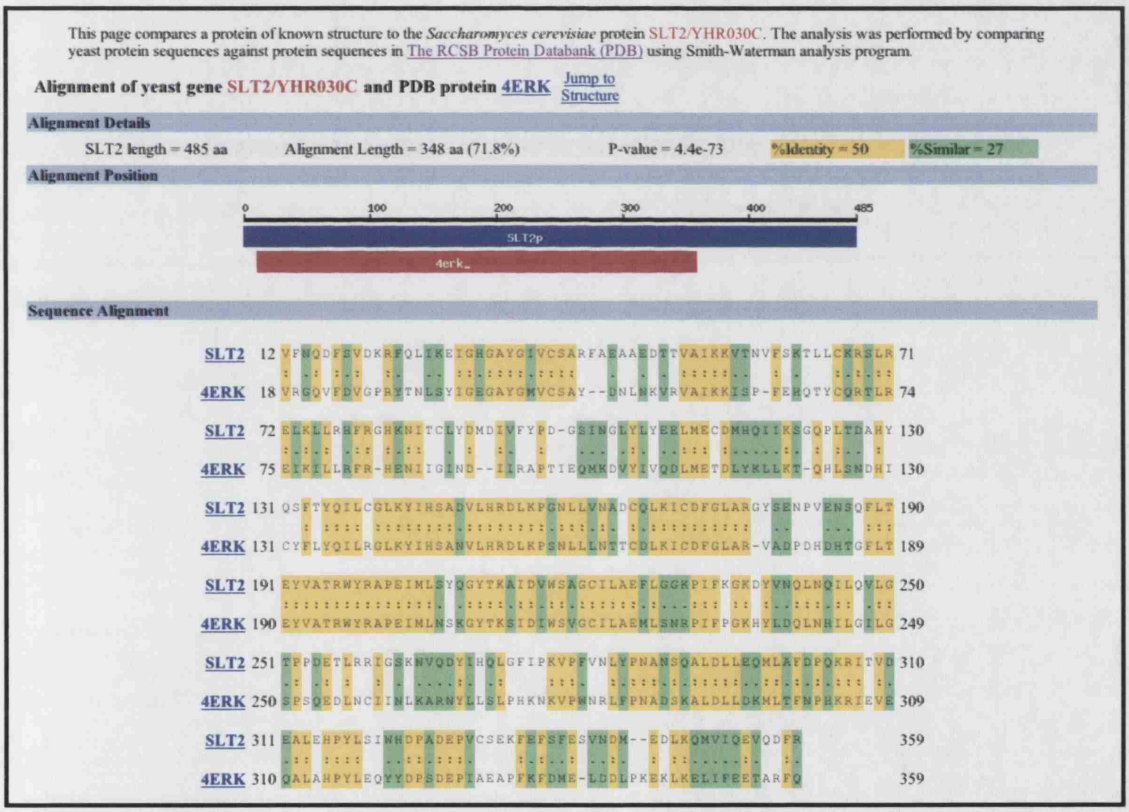


Figure 5.2)

A) The five best-matching PDB homologues for the Slt2p MAP kinase.

B) An alignment of N-terminal MAP kinase domain of Slt2p against the Erk2p protein sequence

5.2.3 *Although ERK2 has high sequence identity with the N-terminal domain of Slt2p, modelling suggests slight structural differences*

The Slt2p protein sequence was sent to the SWISS-PROT modelling website for analysis. This algorithm searches for homologous sequences in the protein databank, and using a PDB template calculates with a high degree of accuracy a theoretical protein structure. Using the PDB sequence 4ERK for the modelling a theoretical structure was obtained for the N-terminus MAP kinase catalytic domain of Slt2p (Figure 5.3c).

Looking at the predicted tertiary structure of Slt2p, all of the classic MAP kinase motifs can be seen. This includes the MAP kinase insertion loop, the flexible phosphorylation lip, the ATP and substrate binding regions and the catalytic site required for phosphorylation of downstream substrates. However, there are few clear deviations from the structure in Figure 5.3B. A major difference occurs around the b1L10 region. This is potentially due to the fact that the Slt2p molecule has a large C-terminal tail in close proximity to this region. Another difference is where ATP binds, but is also close to the D-domain, a completely conserved motif essential for binding a variety of molecules including phosphatases and MAP kinase substrates (Tanoue and Nishida, 2003).

5.2.4 *The expression of ERK2 in yeast does not suppress the ts nature of the p82a slt2Δ strain*

From comparison of the two structures in Figures 5.3B,C it is difficult to determine whether ERK2 is functionally identical to Slt2p, so it was decided to place ERK2 MAP kinase under *GPD1* promoter control in expression vector PG-1 (PG1-ERK2). The resultant vector was transformed into p82a and p82a *slt2*Δ.

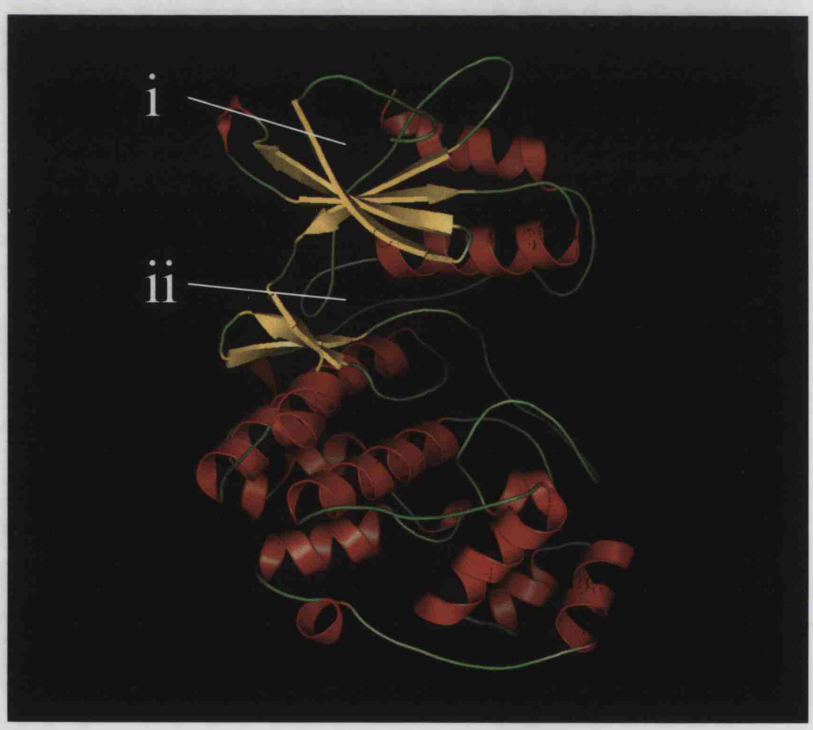
Figure 5.3)

Reference diagram of (A) ERK2 The N-terminal domain (residues 1–109 and 320–358) is formed largely of *b* strands (green) and two helices, C (blue) and *a*L16 (magenta). The C-terminal domain (residues 110–319) is mostly helical (blue), contains the phosphorylation lip (red) and the MAP kinase insertion (magenta, labeled MKI), and is the locus of the P*I*1 site, the catalytic loop (residues Arg-147–152, labeled C loop). The side chains of Thr-183, Tyr-185, pTyr-183, and pTyr-185 are shown. (Reproduced from Canagarajah 1997).

The structure of Erk2p from the crystal co-ordinates (4ERK) (A and B) shows a high degree of homology to the theoretical structure of the N-terminus of Slt2p C) the latter calculated via the SWISS-PROT homology-modelling website, but subtle differences are apparent between the two molecules (i and ii) .

B)

C)



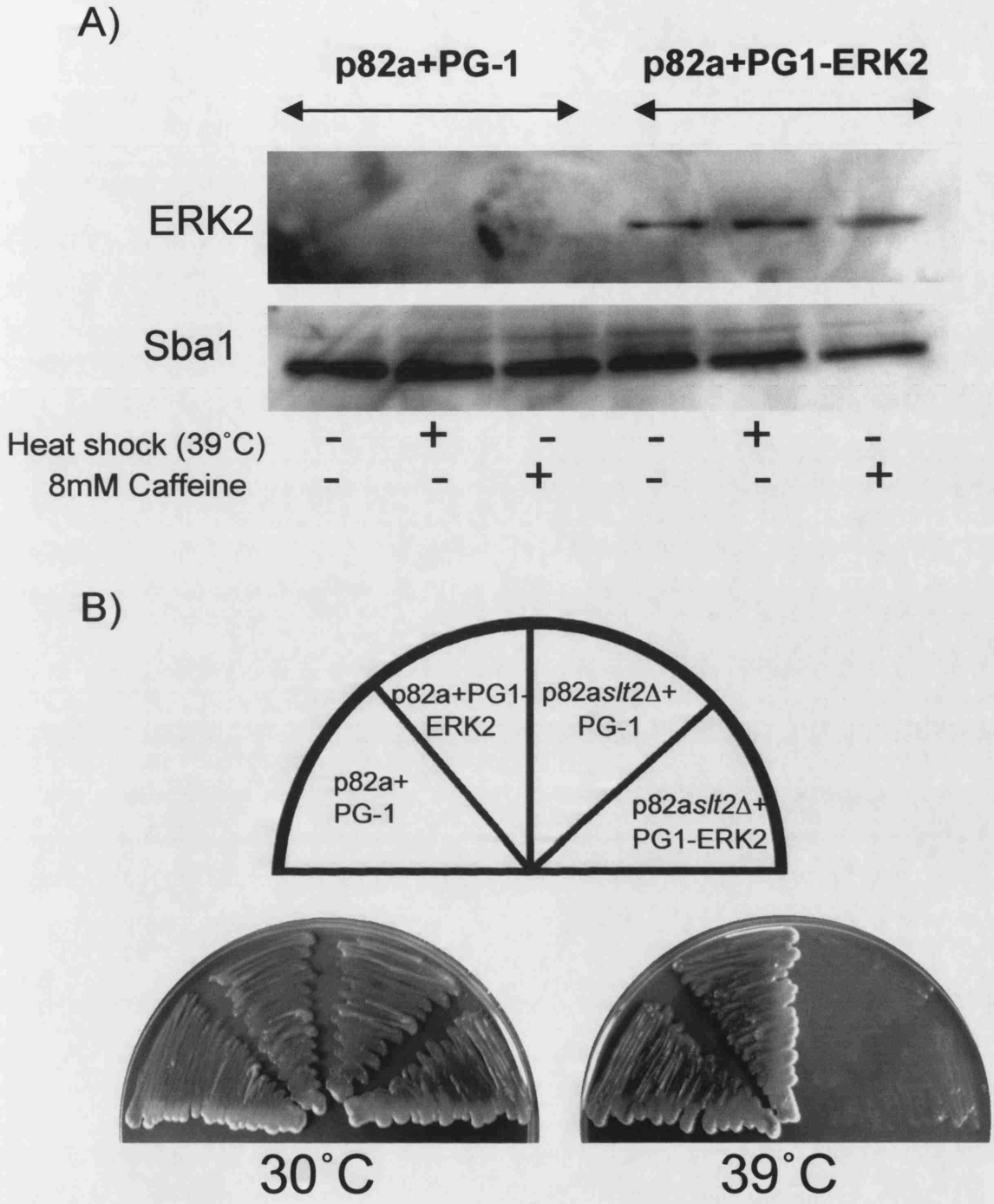


Figure 5.4)

A) Expression of Erk2p in yeast is constant under stress conditions.

B) Expression of Erk2p in yeast, did not suppress the ***ts*** phenotype of p82a *slt2*Δ. Cells containing either the empty vector (PG-1) or a plasmid for a *GPD1*-promoter regulated expression of Erk2p were plated on media lacking tryptophan and grown 2d at the indicated temperatures.

P82a cells transformed with PG1-ERK2 were grown to mid log and either left at 30°C for 1 h (unstressed), stressed for 1 h at 39°C or grown in medium containing 8mM caffeine for 1h. Total cell lysate was then extracted and probed for ERK2 expression using an anti-ERK2 antibody (Santa Cruz Biotechnologies). As shown (Figure 5.4), ERK2 was consistently expressed under unstressed, heat shock at 39°C for 1h and the addition of 8mM caffeine to the media.

p82a and p82a *slt2Δ* cells transformed with either a control vector (PG-1) or the ERK2 expression vector (PG1-ERK2) were streaked on media lacking tryptophan and grown at either 30°C or 39°C. Whereas cells containing the native *SLT2* gene grew at 39°C, cells lacking this *SLT2* gene did not grow (Figure 5.4), suggesting that ERK2 expression in the p82a *slt2Δ* cells was not conferring Slt2p function.

5.2.5 *BLAST search against S. cerevisiae Slt2p reveals a protein with greater homology, the human MAP kinase, BMK1*

After consideration, it was realised that although a good database, PDB only contains proteins for which a structure has been determined, thus was not comprehensive enough for our search. The protein sequence of Slt2p was BLAST searched against *H. Sapiens* defined ORF's. The top matching ORF was ERK5 isoform 1 (also known as BMK1, its GENBANK accession number being NP_002740). It was decided to test the effect of expressing this protein in the p82a *slt2Δ* strain.

5.2.6 *BMK1 expression in S. cerevisiae rescues the temperature-sensitivity of cells lacking the SLT2 gene*

The BMK1 gene was cloned into the PG-1 expression vector to give PG1-BMK1 (see Section 2.6.6). Analysis of the expression of this protein in *S.cerevisiae* using an anti-

BMK1 antibody (Santa Cruz Biotechnologies) revealed constant levels of expression of the protein under unstressed and stressed conditions (Figure 5.5).

P82a and P82a *slt2*Δ cells transformed with either the control vector (PG-1) or the BMK1expression vector (PG1-BMK1) were streaked on media lacking tryptophan and grown at either 30°C or 39°C. PG1-BMK1 restored high temperature growth to cells lacking the native *SLT2* gene (Figure 5.5), suggesting that BMK1 expression in the P82a *slt2*Δ cells was providing substantial Slt2p function.

5.2.7 BMK1 is able to activate Rlm1p in yeast

Although we had shown that the temperature sensitivity of P82a slt2Δ cells was suppressed by expressing of BMK1, it was not known whether this was through Rlm1p activation. To investigate this, the plasmid reporter of Rlm1p-activity (*YIL117c-LacZ*, (Jung et al., 2002)) was transformed into p82a and p82a *slt2*Δ cells already transformed with either PG-1 or PG-BMK1. As in Chapters 3 and 4, Rlm1p activity in unstressed cells, cells heat shocked at 39°C or cells treated with 8mM caffeine was measured via β-Galactosidase assay (Figure 5.6). While BMK1 expression did not fully restore the Rlm1p activity to wild type levels, this activity was significantly increased above that of the control strain (p82a *slt2*Δ containing PG-1) and displayed a degree of stress activation.

5.2.8 BMK1 binds to Hsp82 and Hsc82 via the yeast two-hybrid system

Having shown that BMK1 expression in yeast can at least partially suppress the defect associated with loss of Slt2p function by activating Rlm1p, we decided to investigate whether BMK1 could bind the yeast isoforms of Hsp90, Hsp82 and Hsc82. A PJ694-a strain expressing an AD-BMK1 fusion was generated for yeast two-hybrid purposes. This strain was mated against the PJ694-α strains expressing either Hsp82-BD or Hsc82-BD and the resulting diploids were stressed as previously, to measure BMK1:Hsp82/Hsc82 interaction via β-Galactosidase assay. Interestingly, stressed-reinforced interactions between BMK1 and Hsp82, and between BMK1 and Hsc82 were apparent (Figure 5.7). Just as with the binding of Slt2p and Hsp82, the caffeine

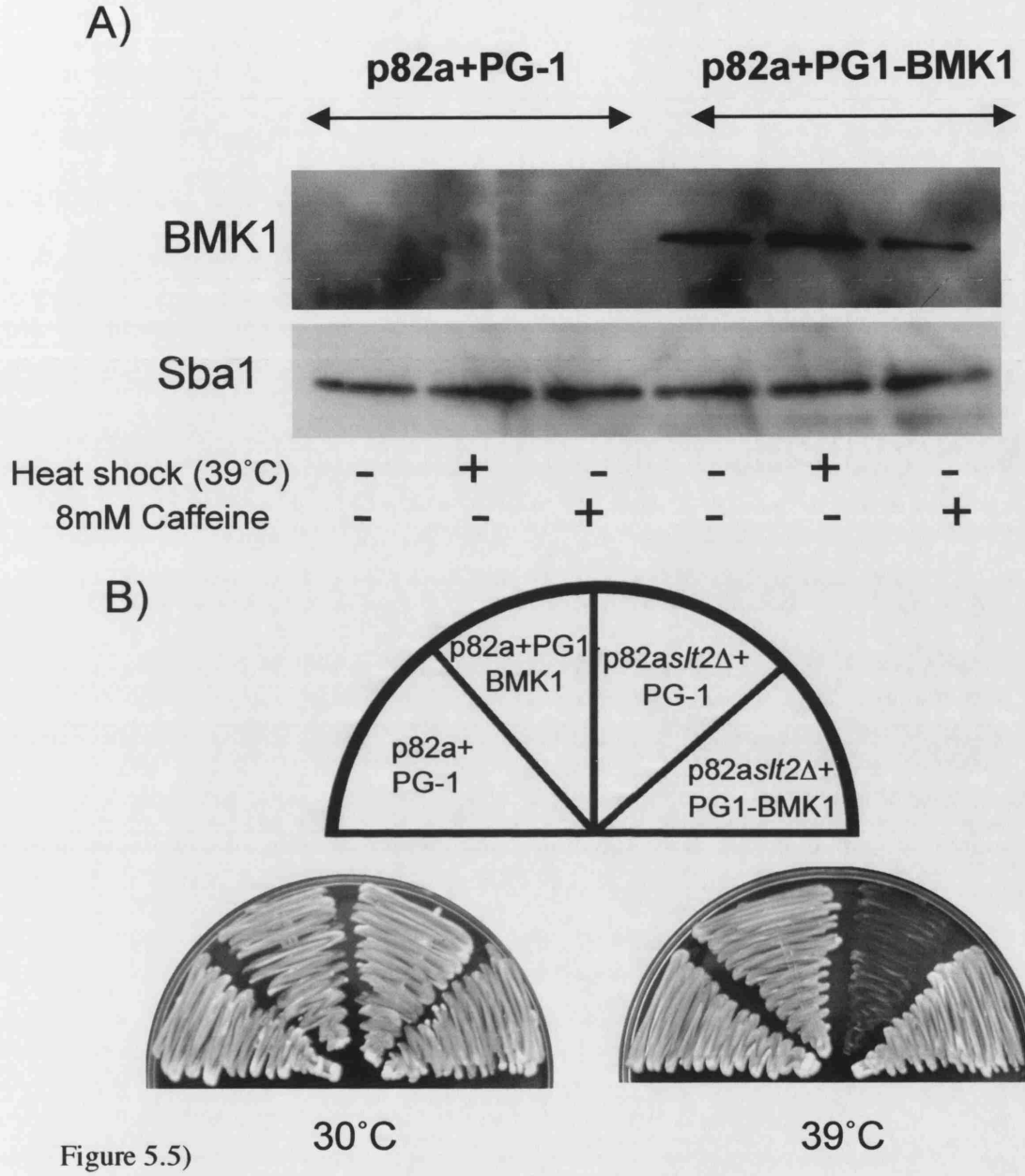


Figure 5.5)

A) Expression of Bmk1p in yeast from vector PG1-BMK1is constant under stress conditions.

B) BMK1 expression efficiently suppressed the *ts* phenotype of p82a *slt2*Δ. Cells containing either the empty vector (PG-1) or a plasmid for a *GPD*-promoter regulated expression of Bmk1p were plated on media lacking tryptophan and grown 2d at the indicated temperatures.

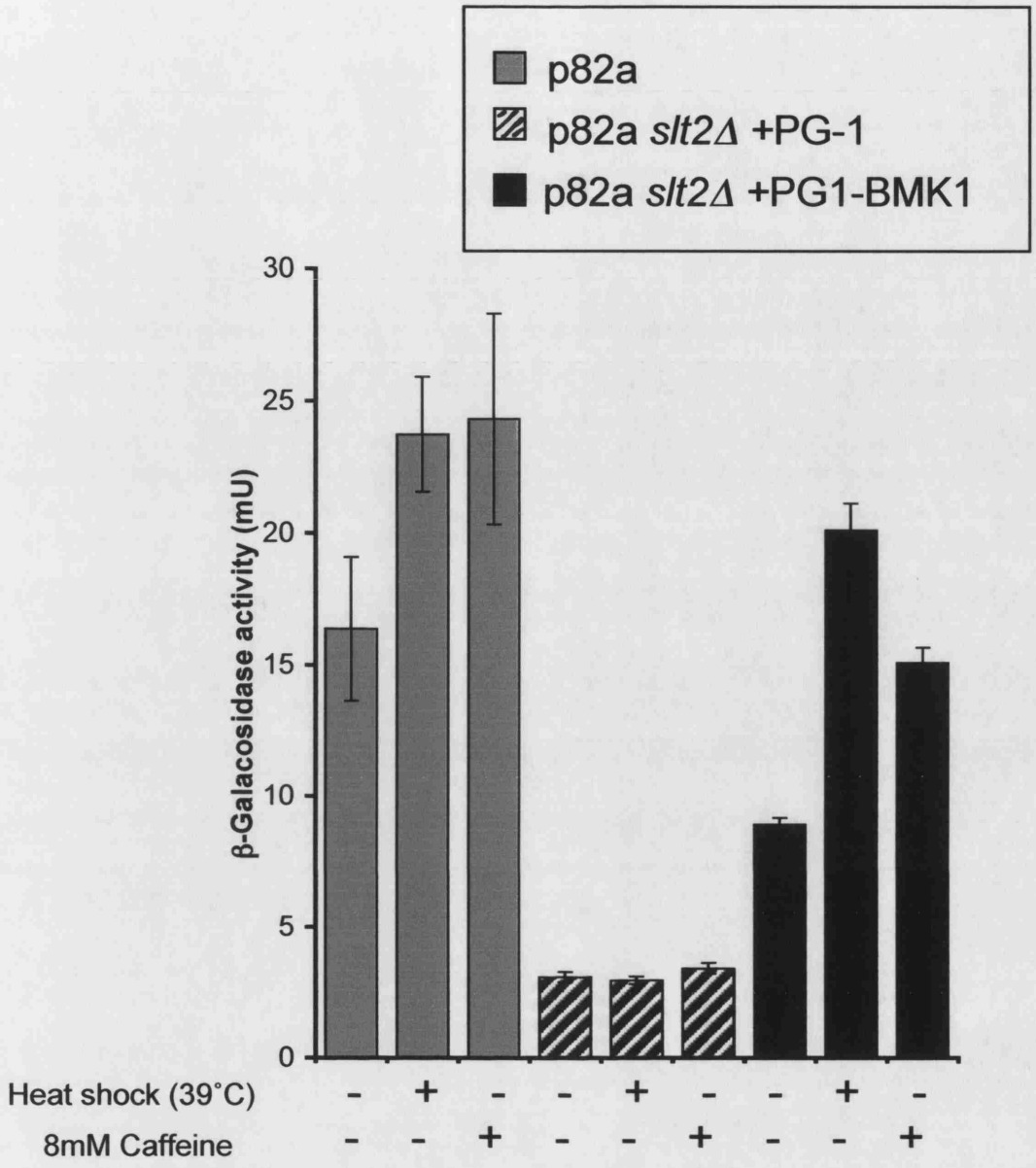


Figure 5.6)

The expression of BMK1 in cells lacking Slt2p allows a partial recovery of basal and stress-induced Rlm1p activity. Cells were grown and stressed as in in Figs. 4.14, 4.16. Rlm1p activity was measured via the *YIL117c*-promoter-*LacZ* reporter plasmid is the mean and SD of three separate experiments.

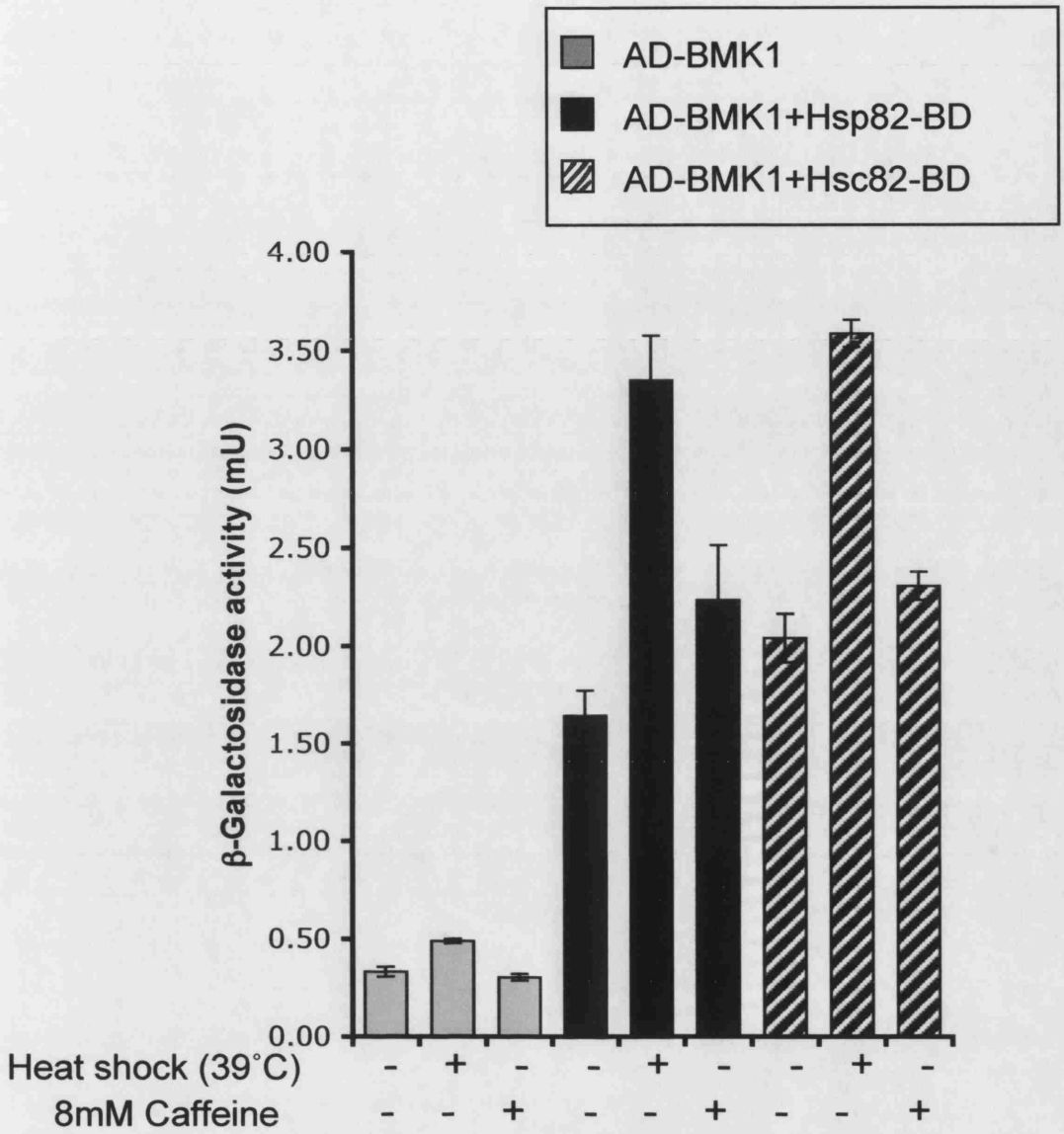


Figure 5.7)

The mammalian MAP kinase BMK1 binds to the yeast Hsp90 isoforms Hsp82 and Hsc82 in the yeast two hybrid system. Cells were stressed as in Figs. 4.14, 4.16. The Hsp82:Slt2p interaction was measured via β-Galactosidase assay and is mean and SD of three separate experiments.

interaction was not so highly enhanced, yet there was a much lower selectivity of BMK1 interaction to the two isoforms of Hsp90, the Hsc82 interaction in this case being as strong as the Hsp82 interaction with BMK1 (Figure 5.7).

5.3 Conclusion

The results in this chapter establish that Slt2p engages in two-hybrid interaction to the human isoforms of Hsp90 in much the same way it interacts with the yeast Hsp82 and Hsc82. However, it is the Hsp90β (non-heat inducible isoform) that interacts with Slt2p more strongly (Figure 5.1). The human MAP kinase, BMK1 can partially compensate for loss of Slt2p in yeast, by activating at least one downstream target of Slt2p, the Rlm1p transcription factor (Figures 5.5, 5.6). This human MAP kinase engages in two-hybrid interactions with both yeast isoforms of Hsp90, Hsp82 and Hsc82, an interaction that like the Slt2p:Hsp82 interaction is a stress-induced one. This is a highly significant result, as it implies that BMK1 might bind the two human isoforms of Hsp90, Hsp90α and Hsp90β in mammalian systems. Clearly, if time had allowed, these interactions would have been extended and confirmed in studies of direct protein binding (as in Chapter 4). In addition, it would have been shown whether interaction was specific for the dually phosphorylated state of BMK1.

It remains to be seen whether BMK1 is being dually phosphorylated under the stress conditions used and whether activation of BMK1 is by Mkk1/2p or is using a different upstream MAP kinase kinase from another pathway, such as the Hog1p pathway. With regards to the BMK1:Hsp82/Hsc82 interaction, some co-chaperones that are not present (or are slightly different) in mammalian systems may be interfering with the interaction seen by the yeast two-hybrid system.

6 Current and future directions of this research

The aim of this study was to understand the cell integrity defect phenotypes generated by mutating Hsp90 and lowering the Hsp90 levels in yeast. Loss of the CTA domain on *S. cerevisiae* HSF (the *HSF (1-583)* mutant) generates a lower sustained HSF activity at all temperatures and the cells also become *ts*, (Jakobsen and Pelham, 1991; Nieto-Sotelo et al., 1990; Sorger, 1990). Almost a full capacity for high temperature growth can be restored to these *HSF (1-583)* mutant cells with overexpression of the Hsp82 isoform of the yeast Hsp90 chaperone (though not an overexpression of the 97% similar Hsc82 (Morano et al., 1999)). The requirement for CTA domain-directed gene expression at high temperatures is therefore the specific need to induce a high level of Hsp82, the strongly heat-inducible isoform of Hsp90.

Chapter 3 reveals that the *ts* phenotype of the *HSF (1-583)* mutant can also be suppressed by osmotic stabilisation or by the overexpression of a constitutively active form of Rlm1p, the transcription factor that reinforces cell integrity gene expression. By using an Rlm1p-activity based assay, loss of stress induction of Rlm1p activity was demonstrated in the HSF CTA truncation mutant, a loss of activity that can be partially restored by Hsp82 overexpression (Figure 3.5). The major function of the CTA domain of yeast HSF appears therefore to be to mediate a strong induction of the Hsp82, necessary for Slt2p to activate the Rlm1p *trans*-activator of cell wall genes. Such a function would be dispensable in the absence of a cell wall, which may be one explanation as to why the HSF possesses only a single transactivator domain in many organisms. It has long been known that yeast needs higher Hsp90 chaperone activity in order to grow at 37-39°C (Borkovich et al., 1989; Morano et al., 1999), but the precise reasons for this requirement have remained a mystery. Originally it was thought that this

increased Hsp90 might merely maintain the equilibria of Hsp90-protein associations in the cell, the increased level of Hsp90 at higher temperatures acting to counteract the weakening of noncovalent interaction as the temperature is raised (Nathan et al., 1997). This study shows this elevated Hsp90 level is essential for stress activation of Rlm1p, needed for the reinforcement of cell integrity gene expression that allows high temperature growth.

In Chapter 4, we examined a set of eight *S. cerevisiae* mutants that are *ts* for growth at 37°C due to point mutations in Hsp82, their sole form of Hsp90 chaperone (Nathan and Lindquist, 1995). Out of the eight mutants six were found to be osmoremedial in regards to their *ts* phenotypes and several were sensitive to growth on caffeine (Figures 4.2, 4.3). The most *ts* mutant of the set, T22I*hsp82* was also the most caffeine sensitive. Despite this, as with the CTA HSF mutant, growth at both high temperature and in the presence of caffeine is restored when the medium is osmotically stabilised. Again, restoration of T22I*hsp82* high temperature growth was seen with the overexpression of a constitutively active Rlm1p.

Both the *HSF (1-583)* and T22I*hsp82* strains display a cell integrity defect, the former only at high temperatures of growth, the latter at all temperatures of growth. In both, the Slt2p molecule acquires activatory dual phosphorylations under stress conditions, but appears to lack the ability to activate Rlm1p. In *HSF(1-583)* this defect is largely rescued by an increase in Hsp82 level, showing that the lack of the HSF CTA results in Hsp82 not being induced enough under heat shock conditions to provide an active Slt2p molecule. This leads to a lack of Rlm1p activity and a weak yeast cell wall. In the case of the T22I*hsp82* mutant, the level of Hsp82 is kept constantly high, but this Hsp82 defect results in lack of Slt2p mediated Rlm1p activation at all temperatures (see below).

Chapter 4 shows, using both yeast two hybrid and IMAC techniques, that Hsp82 physically binds to Slt2p under stress conditions, and by site-directed mutagenesis that this interaction is dependent on the dual Thr190 and Tyr192 phosphorylation of Slt2p. Although the two isoforms of Hsp90 in *S. cerevisiae* (Hsp82 and Hsc82) are almost

identical in sequence, Slt2p shows a preference for binding to Hsp82 (Figure 4.17). The interaction is with the N-terminal MAP kinase domain, required for catalytic activity, the long C-terminal section containing the unusual polyglutamine tract being dispensable for binding Hsp82 (Figure 4.17). The binding of ATP by Hsp82 was shown to be essential for the Slt2p:Hsp82 interaction, since a mutation that abolished ATP binding (D79N*hsp82*) severely reduced Slt2p association (Figure 4.12). Any condition that slowed the ATPase step of the chaperone cycle, for example the addition of molybdate or a mutation in Hsp82 (E33A) strengthened the interaction up to five-fold. Such stabilising conditions of this client:chaperone complex might be useful for future crystallographic studies.

Observing the phenotypic effects of expressing mutant forms of Hsp82 could be considered potentially risky. After all, Hsp90 binds a vast array of client proteins, so a single mutation could affect many Hsp90 interactions, leading to confusion when interpreting the phenotypes resulting from compromised Hsp90 activity. Indeed, this is probably what we are seeing with many of the Lindquist mutants, for example the E381K*hsp82* mutant is slightly caffeine sensitive, but displays relatively normal Rlm1p activity. This mutation occurs in the middle domain of Hsp82, in the client-binding domain. Could the defect arise from a particular client interaction becoming significantly decreased? In the study produced by Harris and co-workers (Harris et al., 2001), the E381K*hsp82* mutant was shown to display an enhanced HSF activity (as detected by measuring HSE-*LacZ* levels) which may have particular relevance to our work. We have been fortunate to get such clear-cut phenotypes from both the *HSF(1-583)* and T22I*hsp82* mutants.

What is the nature of the Slt2p:Hsp82 interaction presumably needed for normal Slt2p function? There are several possibilities. It is well described that dual phosphorylation of MAP kinases (for example ERK2 and p38 in humans) results in their homodimerisation and nuclear accumulation (Khokhlatchev et al., 1998). The region required for this dimerisation (a leucine-rich region in ERK2) is not well conserved in Slt2p, so it could be suggested that the binding of Hsp90 (itself a dimer) assists in

allowing two Slt2p monomers to associate to form an active dimeric MAP kinase. It could be this difference in the region of dimerisation that decides which MAP kinases are Hsp90-dependent. Another possibility is that the Slt2p molecule is fully capable of phosphorylation and homodimerisation, but is unable to translocate efficiently to the nucleus to activate its downstream targets without the assistance of Hsp82. Slt2p tagged with green fluorescent protein (GFP) in the T22I*hsp82* mutant appears to show a lack of stress-induced nuclear accumulation (data not shown), but as with all work on MAP kinase nuclear shuttling, the data is ambiguous. For example, an Slt2p molecule incapable of dimerisation could also potentially be unable to enter the nucleus under stress conditions. Current work in our lab suggests Hsp82 may have a role in nuclear translocation of a select set of proteins, although the exact nature is still to be uncovered. More likely, the interaction occurs with the fully phosphorylated and dimerised form of Slt2p, and that Hsp90 interaction 'tweaks' the molecular structure of the MAP kinase, either assisting the interaction with the MAP kinase docking site (D-domain) on Rlm1p or allowing the Slt2p to achieve full enzymatic activity after this docking interaction.

One can consider more specifically the nature of the problem with the T22I mutant. Initially it was thought that the interaction of Slt2p with the T22I mutant Hsp82 might be much more transient than with the wild type Hsp82, due to the previous knowledge that purified T22I mutant Hsp82 protein has an accelerated ATPase activity, around five-fold higher than wild type Hsp82 (Prodromou 2000). This would concur with the data shown in Figures 4.12, 4.13, 4.19, that the addition of molybdate or the E33A*hsp82* mutation can significantly strengthen the interaction with Slt2p, whereas the D79N*hsp82* effectively nullifies the interaction. However during the course of the study, *in vivo* interaction was found to be maintained with the T22I*hsp82* mutant protein, indeed it appears that the interaction has become reinforced (Fig 4.19). A simple explanation is that a certain set of co-chaperones is required for the full activity of Slt2p along with the Hsp82, and that there is a binding defect for one or more of these co-chaperones for the T22I*hsp82* protein. This correlates with the finding that the T22I mutant form of Hsp90 cannot bind the co-chaperone P23p (Sba1p) (Prodromou, data unpublished).

Researchers in the past have tried to link the cell integrity pathway with the Hsp90-dependent heat shock response (Kamada et al., 1995; Zu et al., 2001) but appear to have approached the problem the wrong way round; it is not the Slt2p pathway that is regulating the yeast heat shock response, it is HSF-directed heat-induction of an elevated Hsp90 level that is allowing the Slt2p molecule to activate its downstream target Rlm1p (Figure 3.5).

In Chapter 5, it is shown in the human MAP kinase family member most similar to yeast Slt2p, ERK5 (BMK1), can provide Slt2p function in yeast. Up to now, this had not been described, possibly because BMK1 is one of the least well studied MAP kinases of *H. Sapiens*. Future work on the mechanism of Slt2p action, also analysis of BMK1 activity in yeast (see below), may assist in increasing knowledge about this potentially biomedically useful protein. The fact that both Slt2p and BMK1 interact with Hsp82 and Hsc82 suggests there is something in their structures that is potentially unique for binding to Hsp90.

6.1 Possibilities for future experimentation

This work is the first to uncover an Hsp90 requirement in a member of the MAP kinase class of kinases, though Hsp90 is needed for structurally-related kinases (e.g. cyclin-dependent kinases (Munoz and Jimenez, 1999), which like MAP kinases, phosphorylate their substrates at sites with a proline in the n+1 position). Equally, it is also clear Hsp90 is not required for the activities of many MAP kinases (for example, purified recombinant mammalian ERK2 can be rendered active *in vitro* in the absence of Hsp90, either through mutations within ERK2 itself (Emrick et al., 2001), or with co-expression of constitutively active forms of MAP kinase kinase (Khokhlatchev et al., 1997).

Already there is considerable precedent for proteins of *almost* identical structure differing in their dependence on Hsp90 for their activities (e.g. $p60^{v\text{-}src}$ is Hsp90-dependent whereas $p60^{c\text{-}src}$ is not; certain steroid receptors are Hsp90-dependent while others are not; certain mutant p53 forms are Hsp90 requiring, while native p53 appears not to have this

requirement (reviewed in (Csermely et al., 1998; Pearl and Prodromou, 2000; Pratt and Toft, 2003)).

What is not known is how a stress-activated MAP kinase complexes with Hsp90 and how this assists activation of the MAP kinase (or the MAP kinase:docking site interaction). If this can be unravelled this for Slt2p, a small, nonessential, genetically-amenable enzyme of essentially known structure, it will add substantially to our knowledge of how the Hsp90 chaperone activates client proteins.

Demonstrating how altered Hsp90 activity influences a specific protein-protein interaction *in vivo* with altered signalling outcome requires the analysis of a well-characterised pathway. For this, the yeast Slt2p MAP kinase, with its extensive genetics is ideal. Its remarkable 40-50% sequence identity with mammalian MAP kinases allows prediction of the Slt2p monomer and dimer structures to a high degree of confidence using the 2.4Å crystal coordinates of the ERK2 monomer and dimer (Canagarajah et al., 1997). The docking and phosphorylation sites of Slt2p on some of its substrates are also identified e.g. Rlm1p (Jung et al., 2002), and it is possible to model on Slt2p these sites of interaction sites with the MAP kinase kinase activator and with nuclear substrates (from knowledge of the corresponding sites on ERK2 (Chang et al., 2002). The dual phosphorylation of Slt2p by the Mkk1/2p is almost certainly the trigger for release from this Mkk1/2p and dimerisation. We have demonstrated Slt2p dimerisation upon Pkc1p pathway activation by dimethylsuberimidate crosslinking (not shown); also that dimerisation is abolished by the T190A,Y192F mutations rendering Slt2p non-phosphorylatable (Lee et al., 1993). What is not known is how such a stress-activated MAP kinase complexes with Hsp90 or how this facilitates docking site recognition and/or the kinasing of target proteins.

As a result, future work can be carried out in 5 major areas; 1) an investigation into the molecular properties of Slt2p; 2) elucidation of the components of the Hsp82:co-chaperone:Slt2p complex; 3) the physical nature of the Slt2p-Hsp82 interaction, identifying the interacting residues; 4) an *in vitro* reconstitution of the Slt2p:Hsp82:co-

chaperone complex as a model Hsp90-client complex; and 5) investigation of whether it is possible to create a version of the Slt2p molecule that does not require Hsp82 function for full activity.

6.1.1 An investigation into the molecular properties of Slt2p

The initial task will be to express Slt2p in bacteria, both in its non-phosphorylated and its dually phosphorylated form (the form that binds Hsp90 *in vivo*). The latter can be achieved by a co-expression in *E. coli* of this MAP kinase with a constitutively-active form of its upstream MAP kinase kinase (the strategy adopted to obtain sufficient active mammalian ERK2 MAP kinase for structure determination (Khokhlatchev et al., 1997; Khokhlatchev et al., 1998). Thus obtaining pure dually phosphorylated Slt2p will involve co-expressing this MAP kinase in *E. coli* with a constitutively-active form of the upstream MAP kinase kinase (Mkk1/2p), such as the S386P mutant form of the yeast *MKK1* gene (Watanabe et al., 1995).

Once we have shown that we can purify the non-phospho and di-phospho forms of Slt2p (both full length and lacking the C-terminal domain), we can explore some of their molecular properties. This will include an investigation to explore Slt2p dimerisation; is the phosphorylation of the molecule enough to allow spontaneous dimerisation (as occurs with ERK2 (Khokhlatchev et al., 1998)). This will support or dismiss the theory of whether Hsp82 binding assists Slt2p dimerisation. A high resolution crystal structure of non-phospho and di-phopho-Slt2p may assist this analysis and may lead to determination of the structure of the Slt2p in complex with Hsp82.

6.1.2 The elucidation of the full Hsp82:co-chaperone:Slt2p complex

. As described in this study, affinity capture and two hybrid experiments using wild type and mutant forms of Slt2p reveal that Hsp90 interacting with just the dually-phosphorylated, stress-activated forms of Slt2p *in vivo*. The *HSF(1-583)* and T22I*hsp82* phenotypes, in turn, provide genetic evidence that Hsp90 is essential for the latter to

activate the downstream Rlm1p target of Slt2p, therefore signal transduction. It will be important to fully characterise the complexes that form *in vivo* between Hsp90 and this dually phosphorylated model MAP kinase, in order to subsequently reconstitute them in *vitro* and later determine the structural changes induced by the chaperone.

Characterisation of the complexes that form *in vivo* between Hsp90 and the dually phosphorylated model MAP kinase can use the proven technology of tandem affinity-purification (TAP) 'tagging' of individual components in multi-protein complexes (Rigaut et al., 1999). The TAP-tag encodes a calmodulin binding peptide and protein A, separated by a TEV protease cleavage site. By integrative transformation of yeast this tag is fused to either the N- or the C-terminus of the protein of interest. The fusion protein expressed in the yeast is then purified by two successive affinity columns.

The artefacts that frequently result when one component of a complex is substantially over-expressed, can be avoided by TAP-tagging single copy genes under the control of their native promoters, thus helping to ensure native 'dosage' of the tagged component with respect to the other components of the target complex. To this end Slt2p can be N- and C-terminally TAP-tagged Slt2p in cells that express either wild type or T22I mutant Hsp90. One can also express in such cells, the control non-phosphorylatable and kinetically-dead versions of TAP-Slt2p, then prepare protein extracts from cells with and without treatments that activate Slt2p (heat or caffeine stress). These extracts can then be used with an efficient system for TAP-based affinity-capture, the isolated TAP-tagged complexes being subjected to detailed analysis. The latter will generally entail an additional size fractionation under non-denaturing conditions, such downstream purification by gel filtration and/or gradient centrifugation feeding parallel compositional analysis by gels and mass spectrometry.

It will be informative to see if the analyses can reveal: (i) the step defective in the T22I*hsp82* mutant; (ii) the effects of inhibition of the chaperone with Hsp90-targetting drugs (geldanamycin and radicicol, inhibitors of ATP binding and Hsp90 N-terminal domain dimerisation (Prodromou et al., 2000) in the chaperone cycle); and (iii) the

effects of stabilising the MAP kinase:Hsp90 complex with molybdate (an inhibitor of the final ATPase step of the Hsp90 cycle (Hartson et al., 1999; Pratt, 1997)). This should reveal in a biological context the effects of inhibiting complex formation at different stages of the Hsp90 cycle, or in T22I*hsp82* mutant background.

6.1.3 In vitro reconstitution of the Slt2p:Hsp82-co-chaperone complex

It is thought client proteins interact largely with the middle domain of Hsp90 (Meyer et al., 2003), but this is not an interaction understood in molecular detail for any client-Hsp90 combination. Establishing the stoichiometry of the Hsp90:active MAP kinase complexes forming *in vivo* will be a necessary first step before initiating the reconstitution of this complex *in vitro*, leading to a capability to produce complexes in the amounts and consistent stoichiometry suitable for structural studies. Certain protein complexes can be reconstituted *in vitro* by mixing in the appropriate stoichiometry the separately-purified interacting components.

Such *in vitro* reconstituted complexes between Tyr/Thr-phosphorylated MAP kinases, Hsp90 and co-chaperones could be studied; (i) by electron microscopy; (ii) by inhibitor studies: to see if they are stabilized by the MoO_4^{2-} anion, or disrupted by ADP/ATP-mimetic Hsp90-specific inhibitors (e.g. geldanamycin); then finally (iii) by enzyme assay: (an *active* MAP kinase will phosphorylate its selective targets *in vitro*, such that the active complexes should phosphorylate GST-Rlm1p *in vitro* in established assays (Watanabe et al., 1997).

6.1.4 The physical nature of the Slt2p:Hsp82 interaction

In this study, we have shown that *in vivo* binding of the dually Thr^{190}/Tyr^{192} phosphorylated Slt2p to Hsp90 can be readily monitored in the two hybrid system (Chapters 4, 5). It might be possible to employ the reverse two hybrid system to identify MAP kinase mutations that prevent this interaction. Such mutations might universally abolish Thr/Tyr-phosphorylation (or dimerisation) of the MAP kinase, or (more interestingly) might *override* the Hsp90 requirement in activity of this MAP kinase.

This would involve: (i) randomly mutagenising a AD-Slt2p-GFP fusion in the Slt2p coding region using standard methods of error-prone PCR; (ii) the mutagenised plasmids transformed into the MAV103 strain used in reverse two hybrid screening (Endoh et al., 2002); (ii) transformants individually screened to eliminate those that do not encode a full-length fusion (detected as no GFP signal in the yeast); (iv) the remaining fluorescent colonies individually mated to the opposite mating type strain (MAV203) containing a *functional* Hsp90-BD "bait"; (v) the resulting diploids (cells now containing *both* the AD-Slt2p-GFP and Hsp90-BD fusions) screened for those that *lack* the stress-induced interaction; (vi) the mutated AD-Slt2p-GFP vectors from such yeasts re-isolated and their Slt2p regions sequenced.

It would be able to automate - in 96 colony-array format - steps (iv-vi) of these screens, including the stress applications and the *lacZ* expression measurements that measure the strength of interaction, as with the *LacZ* expression measurements used in this study. Mutant MAP kinases may fail to interact with Hsp90, not because of any alteration to their Hsp90-interacting residues, but because they either fail to become dually Thr/Tyr phosphorylated at their TEY motif with MAP kinase pathway activation, or are phosphorylated but fail to dimerise. It will therefore be necessary to show if any non-Hsp90-interacting mutant AD-Slt2-GFP fusions are still phosphorylatable and dimerise. Phosphorylatable fusions can be identified using the anti-mammalian Thr^{202}/Tyr^{204}-p44/42 MAP kinase monoclonal antibody (NEB); while dimerisation could be demonstrated by dimethylsuberimidate crosslinking (both techniques being applied to crude extracts from the yeast used in reverse two hybrid screening, the AD-Slt2p-GFP fusion being readily distinguished from the native Slt2p of this strain by virtue of its larger size).

As in (1) above, mutant forms of AD-Slt2p-GFP that are found to be altered in their interaction with Hsp90 but that are still Thr^{190}/Tyr^{192} phosphorylated with stress could be reintroduced into *slt2Δ* and *mkk1/2Δ*, *slt2Δ*, *T22Ihsp82*, *slt2Δ* and *T22Ihsp82*, *mkk1/2Δ*, *slt2Δ* strains bearing the *YIL117c-LacZ* reporter of Rlm1p activity, to determine whether if they suppress the defective activation of the Rlm1p target of Slt2p in these strains, both in the presence and absence of Mkk1/2p.

6.1.5 Investigating whether it is possible to create a version of the Slt2p molecule that does not require Hsp82 function for full activity.

Constitutively-active, gain-of-function (GOF), and oncogenic mutants of MAP kinases have already proved to be valuable tools. Such GOF mutations can be *directly selected* in yeast (e.g. for Fus3p (Emrick et al., 2001) and Hog1p (Yaakov et al., 2003). It has though proved much more difficult to obtain active mutant forms of mammalian MAP kinases, with properties similar to the physiologically active, dually Thr/Tyr-diphosphorylated forms. The only success to date has been by: first introducing the GOF mutations originally identified for yeast Fus3p into rat ERK2 (all at residues precisely conserved between Fus3p and ERK2 (Emrick et al., 2001)); then observing the effects of these mutations on: (i) kinase and autophosphorylation activities of a bacterially-expressed ERK2; (ii) the ability of these mutant ERK2s to phosphorylate known physiological targets when transfected back into mammalian cells. Such a strategy is possible because MAP kinase structure is so tightly conserved. It also shows just how valuable *yeast* screens for altered MAP kinase activity can be when engineering altered activity into *mammalian* MAP kinases (where GOF mutations can be associated with malignancy (Ikeda et al., 1997)).

Thus our work, described above, indicates that yeast systems might allow strong, direct *in vivo* selection for some of the following classes of mutation (and possibly all three): 1) Gain of function (GOF) mutations in Slt2p, 2) Slt2p mutations leading to a loss of Hsp90 dependence of Slt2p stress-activated MAP kinase signalling and 3) Slt2p mutations altering the interaction of the dually Thr^{190}/Tyr^{192} phosphorylated Slt2p with Hsp90 in the two hybrid system.

A Gal4p AD-Slt2p fusion is functional, does not self-activate in the 2-hybrid system, and interacts with Hsp90. For a mutagenesis study this fusion could be placed under an inducible (e.g. *MET25*) promoter (so as to detect toxic effects of extreme Slt2p activity), also give a C-terminal-6xHis tail (so as to allow detection of Hsp90 association by both

the two-hybrid and nickel resin retention). Standard methods of site-directed mutagenesis could be used to introduce, singly and in combination, those mutations that are known to cause GOF in other yeast MAP kinases. 7 such mutations have so far been identified for Fus3p (Emrick et al., 2001) and 8 for Hog1p (Yaakov 2003). The effects of introducing such mutations into the protein could then be studied, by observing the Rlm1p activity in both *slt2*Δ and *bck1*Δ cells, basal and stress-induced levels of phosphorylation of each mutated form of Slt2p and the effect of introducing the mutations into the Slt2p protein on the strength of binding to Hsp82.

Study of the transformants mentioned above will reveal to what extent an induction of mutant Slt2p forms can suppress the defective activation of an Slt2p target in these strains (or, if highly active, cause toxicity). This will indicate whether these mutant forms are active MAP kinases, both in the presence and in absence of the upstream MAP kinase kinase. Mutations that suppress in the *absence* of the upstream MAP kinase kinase (Mkk1/2p) are most probably those that significantly enhance rates of intramolecular autophosphorylation (as with certain mutants of ERK2 (Emrick et al., 2001)).

The above studies should show if constitutive activation of Slt2p *invariably* leads to Hsp90 independence, or if it is possible to render the Slt2p activity independent of Hsp90 *in vivo*. How the mutations identified in the above studies affect the MAP kinase monomer and dimer structures (Canagarajah et al., 1997) could be examined by incorporating these point mutations by homology modelling into the X-ray structure of ERK2. Thus it should be possible to rationalise the kinase and autophosphorylation activities of mutant Slt2p forms in the above assays in terms of the relative proximity of the phosphoacceptor and nucleophilic groups. In addition, if mutations render activity independent of Hsp90, it will be interesting to also incorporate these by homology modelling; then apply the same approach; also to identify if these residues are different in the Hsp90-independent ERK2 MAP kinase (this may reveal key features needed for Hsp90-dependence).

As mentioned previously these studies have provided the first example of Hsp90 binding to a protein of the MAP kinase class. Structural studies with other kinase molecules binding to Hsp90 have not been fruitful and potentially this interaction may provide the added insight required. Most Hsp90 client proteins become unstable and are degraded when Hsp90 function is compromised. This appears not to be so with Slt2p. No evidence of an increase in degradation of this protein is ever seen in the T22I*hsp82* mutant or the *HSF (1-583)* mutant strains or following the addition of radicicol. Another novel aspect of the interaction is the selected binding of Hsp82 to the dually phosphorylated and therefore at least partially active form of Slt2p. Hsp90 usually binds to its clients keeping them stable, and releasing them when they become activated. Studies carried out on Hsp90:MAP kinase interactions e.g. (Miyata et al., 2001) state categorically that ERK2 does not interact with Hsp90, yet it may be that the interaction is not being measured with the dually phosphorylated form of ERK2, and the stimulated interaction is being overlooked. The Miyata study (Miyata et al., 2001) shows that a larger family of MAP kinase related molecules exist than previously thought, and that some of these molecules indeed interact with Hsp90. It has not investigated however whether their interaction is with the activated or non-activated form.

Overall the work carried out in this study and potential future work described above suggests that the yeast Slt2p stress-activated MAP kinase has a tremendous potential to provide unique insights into structure/activity relationships and Hsp90 dependence within this kinase family.

7 References

Abe, J., Kusuhara, M., Ulevitch, R. J., Berk, B. C., and Lee, J. D. (1996). Big mitogen-activated protein kinase 1 (BMK1) is a redox-sensitive kinase. J Biol Chem *271*, 16586-16590.

Adams, A., Gottschling, D. E., Kaiser, C. A., and Stearns, T. (1997). Methods in Yeast Genetics. (Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press).

Ahn, S. G., and Thiele, D. J. (2003). Redox regulation of mammalian heat shock factor 1 is essential for Hsp gene activation and protection from stress. Genes Dev *17*, 516-528.

Bell, M., and Engelberg, D. (2003). Phosphorylation of Tyr-176 of the yeast MAPK Hog1/p38 is not vital for Hog1 biological activity. J Biol Chem *278*, 14603-14606.

Bonner, J. J., Carlson, T., Fackenthal, D. L., Paddock, D., Storey, K., and Lea, K. (2000). Complex regulation of the yeast heat shock transcription factor. Mol Biol Cell *11*, 1739-1751.

Borkovich, K. A., Farrelly, F. W., Finkelstein, D. B., Taulien, J., and Lindquist, S. (1989). hsp82 is an essential protein that is required in higher concentrations for growth of cells at higher temperatures. Mol Cell Biol *9*, 3919-3930.

Breter, H. J., Ferguson, J., Peterson, T. A., and Reed, S. I. (1983). Isolation and transcriptional characterization of three genes which function at start, the controlling event of the *Saccharomyces cerevisiae* cell division cycle: CDC36, CDC37, and CDC39. Mol Cell Biol *3*, 881-891.

Brown, A. J. P., and Tuite, M. F. (1998). Yeast Gene Analysis, Vol 26 (San Diego, Academic Press).

Canagarajah, B. J., Khokhlatchev, A., Cobb, M. H., and Goldsmith, E. J. (1997). Activation mechanism of the MAP kinase ERK2 by dual phosphorylation. Cell *90*, 859-869.

Chang, C. I., Xu, B. E., Akella, R., Cobb, M. H., and Goldsmith, E. J. (2002). Crystal structures of MAP kinase p38 complexed to the docking sites on its nuclear substrate MEF2A and activator MKK3b. Mol Cell *9*, 1241-1249.

Chen, Y., Barlev, N. A., Westergaard, O., and Jakobsen, B. K. (1993). Identification of the C-terminal activator domain in yeast heat shock factor: independent control of transient and sustained transcriptional activity. Embo J *12*, 5007-5018.

Cheng, L., Hirst, K., and Piper, P. W. (1992). Authentic temperature-regulation of a heat shock gene inserted into yeast on a high copy number vector. Influences of overexpression of HSP90 protein on high temperature growth and thermotolerance. Biochim Biophys Acta *1132*, 26-34.

Costigan, C., Gehrung, S., and Snyder, M. (1992). A synthetic lethal screen identifies SLK1, a novel protein kinase homolog implicated in yeast cell morphogenesis and cell growth. Mol Cell Biol *12*, 1162-1178.

Csermely, P., Schnaider, T., Soti, C., Prohaszka, Z., and Nardai, G. (1998). The 90kDa molecular chaperone family: structure, function and clinical applications. A comprehensive review. Pharmacol Ther *79*, 1-39.

Davenport, K. R., Sohaskey, M., Kamada, Y., Levin, D. E., and Gustin, M. C. (1995). A second osmosensing signal transduction pathway in yeast. Hypotonic shock activates the PKC1 protein kinase-regulated cell integrity pathway. J Biol Chem *270*, 30157-30161.

de Nobel, H., Ruiz, C., Martin, H., Morris, W., Brul, S., Molina, M., and Klis, F. M. (2000a). Cell wall perturbation in yeast results in dual phosphorylation of the Slt2/Mpk1 MAP kinase and in an Slt2-mediated increase in FKS2-lacZ expression, glucanase resistance and thermotolerance. Microbiology *146*, 2121-2132.

de Nobel, H., van Den Ende, H., and Klis, F. M. (2000b). Cell wall maintenance in fungi. Trends Microbiol *8*, 344-345.

Delley, P. A., and Hall, M. N. (1999). Cell wall stress depolarizes cell growth via hyperactivation of RHO1. J Cell Biol *147*, 163-174.

Dugyala, R. R., Claggett, T. W., Kimmel, G. L., and Kimmel, C. A. (2002). HSP90alpha, HSP90beta, and p53 expression following in vitro hyperthermia exposure in gestation day 10 rat embryos. Toxicol Sci *69*, 183-190.

Duina, A. A., Kalton, H. M., and Gaber, R. F. (1998). Requirement for Hsp90 and a CyP-40-type cyclophilin in negative regulation of the heat shock response. J Biol Chem *273*, 18974-18978.

Emrick, M. A., Hoofnagle, A. N., Miller, A. S., Ten Eyck, L. F., and Ahn, N. G. (2001). Constitutive activation of extracellular signal-regulated kinase 2 by synergistic point mutations. J Biol Chem *276*, 46469-46479.

Endoh, H., Vincent, S., Jacob, Y., Real, E., Walhout, A. J. M., and Vidal, M. (2002). Integrated version of reverse two-hybrid system for the postproteomic era. Meth Enzymol *350*, 525-545.

Erkine, A. M., Magrogan, S. F., Sekinger, E. A., and Gross, D. S. (1999). Cooperative binding of heat shock factor to the yeast HSP82 promoter in vivo and in vitro. Mol Cell Biol *19*, 1627-1639.

Errede, B., Cade, R. M., Yashar, B. M., Kamada, Y., Levin, D. E., Irie, K., and Matsumoto, K. (1995). Dynamics and organization of MAP kinase signal pathways. Mol Reprod Dev *42*, 477-485.

Eustace, B. K., and Jay, D. G. (2004). Extracellular Roles for the Molecular Chaperone, hsp90. Cell Cycle *3*.

Ferrell, J. E., Jr., and Bhatt, R. R. (1997). Mechanistic studies of the dual phosphorylation of mitogen-activated protein kinase. J Biol Chem *272*, 19008-19016.

Gietz, R. D., Schiestl, R. H., Willems, A. R., and Woods, R. A. (1995). Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast *11*, 355-360.

Grammatikakis, N., Vultur, A., Ramana, C. V., Siganou, A., Schweinfest, C. W., Watson, D. K., and Raptis, L. (2002). The role of Hsp90N, a new member of the Hsp90 family, in signal transduction and neoplastic transformation. J Biol Chem *277*, 8312-8320.

Gray, J. V., Ogas, J. P., Kamada, Y., Stone, M., Levin, D. E., and Herskowitz, I. (1997). A role for the Pkc1 MAP kinase pathway of *Saccharomyces cerevisiae* in bud emergence and identification of a putative upstream regulator. Embo J *16*, 4924-4937.

Gruppi, C. M., Zakeri, Z. F., and Wolgemuth, D. J. (1991). Stage and lineage-regulated expression of two hsp90 transcripts during mouse germ cell differentiation and embryogenesis. Mol Reprod Dev *28*, 209-217.

Gupta, R. S. (1995). Phylogenetic analysis of the 90 kD heat shock family of protein sequences and an examination of the relationship among animals, plants, and fungi species. Mol Biol Evol *12*, 1063-1073.

Gustin, M. C., Albertyn, J., Alexander, M., and Davenport, K. (1998). MAP kinase pathways in the yeast *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev *62*, 1264-1300.

Hahn, J. S., Hu, Z., Thiele, D. J., and Iyer, V. R. (2004). Genome-wide analysis of the biology of stress responses through heat shock transcription factor. Mol Cell Biol *24*, 5249-5256.

Hahn, J. S., and Thiele, D. J. (2002). Regulation of the *Saccharomyces cerevisiae* Slt2 kinase pathway by the stress-inducible Sdp1 dual specificity phosphatase. J Biol Chem *28-37*, 28.

Hahn, J. S., and Thiele, D. J. (2004). Activation of the *Saccharomyces cerevisiae* heat shock transcription factor under glucose starvation conditions by Snf1 protein kinase. J Biol Chem *279*, 5169-5176.

Harris, N., MacLean, M., Hatzianthis, K., Panaretou, B., and Piper, P. W. (2001). Increasing the stress resistance of *Saccharomyces cerevisiae*, by the overactivation of the heat shock response that results from Hsp90 defects, does not extend replicative life span but can be associated with a slower chronological ageing of nondividing cells. Mol Gen Genomics *265*, 258-263.

Harrison, J. C., Bardes, E. S. G., Ohya, Y., and Lew, D. J. (2001). A role for the Pkc1p/Mpk1p kinase cascade in the morphogenesis checkpoint. Nature Cell Biol *3*, 417-420.

Hartson, S. D., Thulasiraman, V., Huang, W., Whitesell, L., and Matts, R. L. (1999). Molybdate inhibits hsp90, induces structural changes in its C-terminal domain, and alters its interactions with substrates. Biochemistry *38*, 3837-3849.

Hayashi, M., Kim, S. W., Imanaka-Yoshida, K., Yoshida, T., Abel, E. D., Eliceiri, B., Yang, Y., Ulevitch, R. J., and Lee, J. D. (2004). Targeted deletion of BMK1/ERK5 in adult mice perturbs vascular integrity and leads to endothelial failure. J Clin Invest *113*, 1138-1148.

Hilscher, W. (1974). [Kinetics of prespermatogenesis and spermatogenesis]. Verh Anat Ges *68*, 39-62.

Hjorth-Sorensen, B., Hoffmann, E. R., Lissin, N. M., Sewell, A. K., and Jakobsen, B. K. (2001). Activation of heat shock transcription factor in yeast is not influenced by the levels of expression of heat shock proteins. Mol Microbiol *39*, 914-923.

Hohmann, S. (2002). Osmotic stress signaling and osmoadaptation in yeasts. Microbiol Mol Biol Rev *66*, 300-372.

Hoj, A., and Jakobsen, B. K. (1994). A short element required for turning off heat shock transcription factor: evidence that phosphorylation enhances deactivation. Embo J *13*, 2617-2624.

Igual, J. C., Johnson, A. L., and Johnston, L. H. (1996). Coordinated regulation of gene expression by the cell cycle transcription factor Swi4 and the protein kinase C MAP kinase pathway for yeast cell integrity. Embo J *15*, 5001-5013.

Ikeda, H., Ohta, N., Furukawa, K., Miyazaki, H., Wang, L., Kuribayashi, K., Old, L. J., and Shiku, H. (1997). Mutated mitogen-activated protein kinase: a tumor rejection antigen of mouse sarcoma. Proc Natl Acad Sci U S A *94*, 6375-6379.

Irie, K., Takase, M., Lee, K. S., Levin, D. E., Araki, H., Matsumoto, K., and Oshima, Y. (1993). MKK1 and MKK2, which encode *Saccharomyces cerevisiae* mitogen-activated protein kinase-kinase homologs, function in the pathway mediated by protein kinase C. Mol Cell Biol *13*, 3076-3083.

Jacoby, J. J., Nilius, S. M., and Heinisch, J. J. (1998). A screen for upstream components of the yeast protein kinase C signal transduction pathway identifies the product of the SLG1 gene. Mol Gen Genet *258*, 148-155.

Jakobsen, B. K., and Pelham, H. R. (1991). A conserved heptapeptide restrains the activity of the yeast heat shock transcription factor. Embo J *10*, 369-375.

James, P., Halladay, J., and Craig, E. A. (1996). Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics *144*, 1425-1436.

Jung, U. S., and Levin, D. E. (1999). Genome-wide analysis of gene expression regulated by the yeast cell wall integrity signalling pathway. Mol Microbiol *34*, 1049-1057.

Jung, U. S., Sobering, A. K., Romeo, M. J., and Levin, D. E. (2002). Regulation of the yeast Rlm1 transcription factor by the Mpk1 cell wall integrity MAP kinase. Mol Microbiol *46*, 781-789.

Kamada, Y., Jung, U. S., Piotrowski, J., and Levin, D. E. (1995). The protein kinase C-activated MAP kinase pathway of *Saccharomyces cerevisiae* mediates a novel aspect of the heat shock response. Genes Dev *9*, 1559-1571.

Kamada, Y., Qadota, H., Python, C. P., Anraku, Y., Ohya, Y., and Levin, D. E. (1996). Activation of yeast protein kinase C by Rho1 GTPase. J Biol Chem *271*, 9193-9196.

Kapteyn, J. C., Van Den Ende, H., and Klis, F. M. (1999). The contribution of cell wall proteins to the organization of the yeast cell wall. Biochim Biophys Acta *1426*, 373-383.

Kasler, H. G., Victoria, J., Duramad, O., and Winoto, A. (2000). ERK5 is a novel type of mitogen-activated protein kinase containing a transcriptional activation domain. Mol Cell Biol *20*, 8382-8389.

Ketela, T., Green, R., and Bussey, H. (1999). *Saccharomyces cerevisiae* mid2p is a potential cell wall stress sensor and upstream activator of the PKC1-MPK1 cell integrity pathway. J Bacteriol *181*, 3330-3340.

Khokhlatchev, A., Xu, S., English, J., Wu, P., Schaefer, E., and Cobb, M. H. (1997). Reconstitution of mitogen-activated protein kinase phosphorylation cascades in bacteria. Efficient synthesis of active protein kinases. J Biol Chem *272*, 11057-11062.

Khokhlatchev, A. V., Canagarajah, B., Wilsbacher, J., Robinson, M., Atkinson, M., Goldsmith, E., and Cobb, M. H. (1998). Phosphorylation of the MAP kinase ERK2 promotes its homodimerization and nuclear translocation. Cell *93*, 605-615.

Laemmli, U. K., Molbert, E., Showe, M., and Kellenberger, E. (1970). Form-determining function of the genes required for the assembly of the head of bacteriophage T4. J Mol Biol *49*, 99-113.

Lagorce, A., Hauser, N. C., Labourdette, D., Rodriguez, C., Martin-Yken, H., Arroyo, J., Hoheisel, J. D., and Francois, J. (2003). Genome-wide Analysis of the Response to Cell Wall Mutations in the Yeast *Saccharomyces cerevisiae*. J Biol Chem *278*, 20345-20357.

Lee, J. D., Ulevitch, R. J., and Han, J. (1995). Primary structure of BMK1: a new mammalian map kinase. Biochem Biophys Res Commun *213*, 715-724.

Lee, K. S., Irie, K., Gotoh, Y., Watanabe, Y., Araki, H., Nishida, E., Matsumoto, K., and Levin, D. E. (1993). A yeast mitogen-activated protein kinase homolog (Mpk1p) mediates signalling by protein kinase C. Mol Cell Biol *13*, 3067-3075.

Lee, K. S., and Levin, D. E. (1992). Dominant mutation in a gene encoding a putative protein kinase (*BCK1*) bypass the requirement for a *Saccharomyces cerevisiae* protein kinase C homolog. Mol Cell Biol *12*, 172-182.

Lee, P., Shabbir, A., Cardozo, C., and Caplan, A. J. (2004). Sti1 and Cdc37 can stabilize Hsp90 in chaperone complexes with a protein kinase. Mol Biol Cell *15*, 1785-1792.

Lee, S., Carlson, T., Christian, N., Lea, K., Kedzie, J., Reilly, J. P., and Bonner, J. J. (2000). The yeast heat shock transcription factor changes conformation in response to superoxide and temperature. Mol Biol Cell *11*, 1753-1764.

Lele, Z., Hartson, S. D., Martin, C. C., Whitesell, L., Matts, R. L., and Krone, P. H. (1999). Disruption of zebrafish somite development by pharmacologic inhibition of Hsp90. Dev Biol *210*, 56-70.

Levin, D. E., Bowers, B., Chen, C. Y., Kamada, Y., and Watanabe, M. (1994). Dissecting the protein kinase C/MAP kinase signalling pathway of *Saccharomyces cerevisiae*. Cell Mol Biol Res *40*, 229-239.

Levin, D. E., Fields, F. O., Kunisawa, R., Bishop, J. M., and Thorner, J. (1990). A candidate protein kinase C gene, PKC1, is required for the *S. cerevisiae* cell cycle. Cell *62*, 213-224.

Ling, M., Merante, F., and Robinson, B. H. (1995). A rapid and reliable DNA preparation method for screening a large number of yeast clones by polymerase chain reaction. Nucl Acids Res *23*, 4924-4925.

Liu, X. D., and Thiele, D. J. (1996). Oxidative stress induced heat shock factor phosphorylation and HSF-dependent activation of yeast metallothionein gene transcription. Genes Dev *10*, 592-603.

Lotz, G. P., Lin, H., Harst, A., and Obermann, W. M. (2003). Aha1 binds to the middle domain of Hsp90, contributes to client protein activation, and stimulates the ATPase activity of the molecular chaperone. J Biol Chem *278*, 17228-17235.

Louvion, J. F., Abbas-Terki, T., and Picard, D. (1998). Hsp90 is required for pheromone signaling in yeast. Mol Biol Cell *9*, 3071-3083.

Louvion, J. F., Warth, R., and Picard, D. (1996). Two eukaryote-specific regions of Hsp82 are dispensable for its viability and signal transduction functions in yeast. Proc Natl Acad Sci U S A *93*, 13937-13942.

MacLean, M., and Picard, D. (2003). Cdc37 goes beyond Hsp90 and kinases. Cell Stress Chaperones *8*, 114-119.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1989). Molecular Cloning, A laboratory Manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory press.

Martin, H., Arroyo, J., Sanchez, M., Molina, M., and Nombela, C. (1993). Activity of the yeast MAP kinase homologue Slt2 is critically required for cell integrity at 37 degrees C. Mol Gen Genet *241*, 177-184.

Martin, H., Rodriguez-Pachon, J. M., Ruiz, C., Nombela, C., and Molina, M. (2000). Regulatory mechanisms for modulation of signaling through the cell integrity Slt2-mediated pathway in *Saccharomyces cerevisiae*. J Biol Chem *275*, 1511-1519.

Maruya, M., Sameshima, M., Nemoto, T., and Yahara, I. (1999). Monomer arrangement in HSP90 dimer as determined by decoration with N and C-terminal region specific antibodies. J Mol Biol *285*, 903-907.

Mazur, P., Morin, N., Baginsky, W., el-Sherbeini, M., Clemas, J. A., Nielsen, J. B., and Foor, F. (1995). Differential expression and function of two homologous subunits of yeast 1,3-beta-D-glucan synthase. Mol Cell Biol *15*, 5671-5681.

Mazzoni, C., Zarov, P., Rambourg, A., and Mann, C. (1993). The SLT2 (MPK1) MAP kinase homolog is involved in polarized cell growth in *Saccharomyces cerevisiae*. J Cell Biol *123*, 1821-1833.

Meyer, P., Prodromou, C., Hu, B., Vaughan, C., Roe, S. M., Panaretou, B., Piper, P. W., and Pearl, L. H. (2003). Structural and functional analysis of the middle segment of hsp90. Implications for ATP hydrolysis and client protein and cochaperone interactions. Mol Cell *11*, 647-658.

Millson, S. H., Truman, A., Wolfram, F., King, V., Panaretou, B., Prodromou, C., Pearl, L. H., and Piper, P. W. (2004). Investigating the protein-protein interactions of the yeast Hsp90 chaperone system by two hybrid analysis; Potential uses and limitations of this approach. Cell Stress Chaperones *9*, (in press).

Millson, S. M., Truman, A., and Piper, P. W. (2003). Vectors for N- or C-terminal positioning of the yeast Gal4p DNA binding or activator domains. BioTechniques *35*, 60-64.

Miyata, Y., Ikawa, Y., Shibuya, M., and Nishida, E. (2001). Specific association of a set of molecular chaperones including HSP90 and Cdc37 with MOK, a member of the MAP kinase superfamily. J Biol Chem *16*, 16.

Miyata, Y., and Yahara, I. (2000). p53-independent association between SV40 large T antigen and the major cytosolic heat shock protein, HSP90. Oncogene *19*, 1477-1484.

Morano, K. A., Santoro, N., Koch, K. A., and Thiele, D. J. (1999). A trans-activation domain in yeast heat shock transcription factor is essential for cell cycle progression during stress. Mol Cell Biol *19*, 402-411.

Morimoto, R. I. (1991). Heat shock: the role of transient inducible responses in cell damage, transformation, and differentiation. Cancer Cells *3*, 295-301.

Morishima, Y., Kanelakis, K. C., Silverstein, A. M., Dittmar, K. D., Estrada, L., and Pratt, W. B. (2000). The Hsp organizer protein hop enhances the rate of but is not essential for glucocorticoid receptor folding by the multiprotein Hsp90-based chaperone system. J Biol Chem *275*, 6894-6900.

Munoz, M. Z., and Jimenez, J. (1999). Genetic interactions between Hsp90 and the Cdc2 mitotic machinery in the fission yeast *Schizosaccharomyces pombe*. Mol Gen Genet *261*, 242-250.

Nathan, D. F., and Lindquist, S. (1995). Mutational analysis of Hsp90 function: interactions with a steroid receptor and a protein kinase. Mol Cell Biol *15*, 3917-3925.

Nathan, D. F., Vos, M. H., and Lindquist, S. (1997). In vivo functions of the *Saccharomyces cerevisiae* Hsp90 chaperone. Proc Natl Acad Sci U S A *94*, 12949-12956.

Nicolet, C. M., and Craig, E. A. (1991). Inducing and assaying heat-shock response in *Saccharomyces cerevisiae*. Methods Enzymol *194*, 710-717.

Nieto-Sotelo, J., Wiederrecht, G., Okuda, A., and Parker, C. S. (1990). The yeast heat shock transcription factor contains a transcriptional activation domain whose activity is repressed under nonshock conditions. Cell *62*, 807-817.

Nonaka, H., Tanaka, K., Hirano, H., Fujiwara, T., Kohno, H., Umikawa, M., Mino, A., and Takai, Y. (1995). A downstream target of RHO1 small GTP-binding protein is PKC1, a homolog of protein kinase C, which leads to activation of the MAP kinase cascade in *Saccharomyces cerevisiae*. Embo J *14*, 5931-5938.

Panaretou, B., Prodromou, C., Roe, S. M., O'Brien, R., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1998). ATP binding and hydrolysis are essential to the function of the Hsp90 molecular chaperone in vivo. Embo J *17*, 4829-4836.

Panaretou, B., Siligardi, G., Meyer, P., Maloney, A., Sullivan, J. K., Singh, S., Millson, S. H., Clarke, P. A., Naaby-Hansen, S., Stein, R., *et al.* (2002). Activation of the ATPase activity of Hsp90 by AHA1 and other co-chaperones. Mol Cell *10*, 1307-1318.

Pearl, L. H., and Prodromou, C. (2000). Structure and in vivo function of Hsp90. Curr Opin Struct Biol *10*, 46-51.

Pearson, G., Robinson, F., Beers Gibson, T., Xu, B. E., Karandikar, M., Berman, K., and Cobb, M. H. (2001). Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev *22*, 153-183.

Philip, B., and Levin, D. E. (2001). Wsc1 and mid2 are cell surface sensors for cell wall integrity signaling that act through rom2, a guanine nucleotide exchange factor for rho1 [In Process Citation]. Mol Cell Biol *21*, 271-280.

Pi, X., Yan, C., and Berk, B. C. (2004). Big mitogen-activated protein kinase (BMK1)/ERK5 protects endothelial cells from apoptosis. Circ Res *94*, 362-369.

Picard, D. (2002). Heat-shock protein 90, a chaperone for folding and regulation. Cell Mol Life Sci *59*, 1640-1648.

Piper, P. W., Millson, S. H., Mollapour, M., Panaretou, B., Siligardi, G., Pearl, L. H., and Prodromou, C. (2003a). Sensitivity to Hsp90-targeting drugs can arise with mutation to the Hsp90 chaperone, cochaperones and plasma membrane ATP binding cassette transporters of yeast. Eur J Biochem *270*, 4689-4695.

Piper, P. W., Panaretou, B., Millson, S. H., Truman, A., Mollapour, M., Pearl, L. H., and Prodromou, C. (2003b). Yeast is selectively hypersensitised to heat shock protein 90 (Hsp90)-targetting drugs with heterologous expression of the human Hsp90, a property that can be exploited in screens for new Hsp90 chaperone inhibitors. Gene *302*, 165-170.

Pratt, W. B. (1997). The role of the hsp90-based chaperone system in signal transduction by nuclear receptors and receptors signaling via MAP kinase. Annu Rev Pharmacol Toxicol *37*, 297-326.

Pratt, W. B., and Toft, D. O. (1997). Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocr Rev *18*, 306-360.

Pratt, W. B., and Toft, D. O. (2003). Regulation of Signaling Protein Function and Trafficking by the hsp90/hsp70-Based Chaperone Machinery. Exp Biol Med *228*, 111-133.

Primig, M., Sockanathan, S., Auer, H., and Nasmyth, K. (1992). Anatomy of a transcription factor important for the start of the cell cycle in *Saccharomyces cerevisiae*. Nature *358*, 593-597.

Prodromou, C., Panaretou, B., Chohan, S., Siligardi, G., O'Brien, R., Ladbury, J. E., Roe, S. M., Piper, P. W., and Pearl, L. H. (2000). The ATPase cycle of Hsp90 drives a molecular 'clamp' via transient dimerization of the N-terminal domains. Embo J *19*, 4383-4392.

Prodromou, C., Roe, S. M., O'Brien, R., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1997). Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. Cell *90*, 65-75.

Prodromou, C., Siligardi, G., O'Brien, R., Woolfson, D. N., Regan, L., Panaretou, B., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1999). Regulation of Hsp90 ATPase activity by tetratricopeptide repeat (TPR)-domain co-chaperones. Embo J *18*, 754-762.

Rajavel, M., Philip, B., Buehrer, B. M., Errede, B., and Levin, D. E. (1999). Mid2 is a putative sensor for cell integrity signaling in *Saccharomyces cerevisiae*. Mol Cell Biol *19*, 3969-3976.

Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Seraphin, B. (1999). A generic protein purification method for protein complex characterization and proteome exploration. Nat Biotechnol *17*, 1030-1032.

Robbins, D. J., and Cobb, M. H. (1992). Extracellular signal-regulated kinases 2 autophosphorylates on a subset of peptides phosphorylated in intact cells in response to insulin and nerve growth factor: analysis by peptide mapping. Mol Biol Cell *3*, 299-308.

Santoro, N., Johansson, N., and Thiele, D. J. (1998). Heat shock element architecture is an important determinant in the temperature and transactivation domain requirements for heat shock transcription factor. Mol Cell Biol *18*, 6340-6352.

Schorr, M., Then, A., Tahirovic, S., Hug, N., and Mayinger, P. (2001). The phosphoinositide phosphatase Sac1p controls trafficking of the yeast Chs3p chitin synthase. Curr Biol *11*, 1421-1426.

Smits, G. J., Kapteyn, J. C., van den Ende, H., and Klis, F. M. (1999). Cell wall dynamics in yeast. Curr Opin Microbiol *2*, 348-352.

Soler, M., Plovins, A., Martin, H., Molina, M., and Nombela, C. (1995). Characterization of domains in the yeast MAP kinase Slt2 (Mpk1) required for functional activity and in vivo interaction with protein kinases Mkk1 and Mkk2. Mol Microbiol *17*, 833-842.

Sorger, P. K. (1990). Yeast heat shock factor contains separable transient and sustained response transcriptional activators. Cell *62*, 793-805.

Sorger, P. K., and Pelham, H. R. (1987). Purification and characterization of a heat-shock element binding protein from yeast. Embo J *6*, 3035-3041.

Sorger, P. K., and Pelham, H. R. (1988). Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation. Cell *54*, 855-864.

Soti, C., Radics, L., Yahara, I., and Csermely, P. (1998). Interaction of vanadate oligomers and permolybdate with the 90-kDa heat-shock protein, Hsp90. Eur J Biochem *255*, 611-617.

Srivastava, P. K., and Amato, R. J. (2001). Heat shock proteins: the 'Swiss Army Knife' vaccines against cancers and infectious agents. Vaccine *19*, 2590-2597.

Tahirovic, S., Schorr, M., Then, A., Berger, J., Schwarz, H., and Mayinger, P. (2003). Role for lipid signaling and the cell integrity MAP kinase cascade in yeast septum biogenesis. Curr Genet *43*, 71-78.

Tamai, K. T., Liu, X., Silar, P., Sosinowski, T., and Thiele, D. J. (1994). Heat shock transcription factor activates yeast metallothionein gene expression in response to heat and glucose starvation via distinct signalling pathways. Mol Cell Biol *14*, 8155-8165.

Tanoue, T., and Nishida, E. (2003). Molecular recognitions in the MAP kinase cascades. Cell Signal *15*, 455-462.

Torres, L., Martin, H., Garcia-Saez, M. I., Arroyo, J., Molina, M., Sanchez, M., and Nombela, C. (1991). A protein kinase gene complements the lytic phenotype of *Saccharomyces cerevisiae* lyt2 mutants. Mol Microbiol *5*, 2845-2854.

Uetz, P., Cagney, G., Lockshon, D., Qureshi-Emili, A., Conover, D., Johnston, M., and Fields, S. (2000). A protein array for genomewide screens of protein-protein interactions. Nature *403*, 623-627.

van Drogen, F., and Peter, M. (2002). Spa2p functions as a scaffold-like protein to recruit the Mpk1p MAP kinase module to sites of polarized growth. Curr Biol *12*, 1698-1703.

Voellmy, R. (2004). On mechanisms that control heat shock transcription factor activity in metazoan cells. Cell Stress Chaperones *9*, 122-133.

Watanabe, Y., Irie, K., and Matsumoto, K. (1995). Yeast RLM1 encodes a serum response factor-like protein that may function downstream of the Mpk1 (Slt2) mitogen-activated protein kinase pathway. Mol Cell Biol *15*, 5740-5749.

Watanabe, Y., Takaesu, G., Hagiwara, M., Irie, K., and Matsumoto, K. (1997). Characterization of a serum response factor-like protein in *Saccharomyces cerevisiae*, Rlm1, which has transcriptional activity regulated by the Mpk1 (Slt2) mitogen-activated protein kinase pathway. Mol Cell Biol *17*, 2615-2623.

Wiech, H., Buchner, J., Zimmermann, R., and Jakob, U. (1992). Hsp90 chaperones protein folding in vitro. Nature *358*, 169-170.

Wolfe, K. H., and Shields, D. C. (1997). Molecular evidence for an ancient duplication of the entire yeast genome. Nature *387*, 708-713.

Workman, P., and Maloney, A. (2002). HSP90 as a new therapeutic target for cancer therapy: the story unfolds. Expert Opin Biol Ther *2*, 3-24.

Yaakov, G., Bell, M., Hohmann, S., and Engelberg, D. (2003). Combination of two activating mutations in one HOG1 gene forms hyperactive enzymes that induce growth arrest. Mol Cell Biol *23*, 4826-4840.

Yamada, T., Hashiguchi, A., Fukushima, S., Kakita, Y., Umezawa, A., Maruyama, T., and Hata, J. (2000). Function of 90-kDa heat shock protein in cellular differentiation of human embryonal carcinoma cells. In Vitro Cell Dev Biol Anim *36*, 139-146.

Yamochi, W., Tanaka, K., Nonaka, H., Maeda, A., Musha, T., and Takai, Y. (1994). Growth site localization of Rho1 small GTP-binding protein and its involvement in bud formation in *Saccharomyces cerevisiae*. J Cell Biol *125*, 1077-1093.

Yan, C., Luo, H., Lee, J. D., Abe, J., and Berk, B. C. (2001). Molecular cloning of mouse ERK5/BMK1 splice variants and characterization of ERK5 functional domains. J Biol Chem *276*, 10870-10878.

Yan, C., Takahashi, M., Okuda, M., Lee, J. D., and Berk, B. C. (1999). Fluid shear stress stimulates big mitogen-activated protein kinase 1 (BMK1) activity in endothelial cells. Dependence on tyrosine kinases and intracellular calcium. J Biol Chem *274*, 143-150.

Young, J. C., and Hartl, F. U. (2000). Polypeptide release by hsp90 involves ATP hydrolysis and is enhanced by the co-chaperone p23. Embo J *19*, 5930-5940.

Young, J. C., Hoogenraad, N. J., and Hartl, F. U. (2003). Molecular chaperones Hsp90 and Hsp70 deliver preproteins to the mitochondrial import receptor Tom70. Cell *112*, 41-50.

Young, J. C., Obermann, W. M., and Hartl, F. U. (1998). Specific binding of tetratricopeptide repeat proteins to the C-terminal 12-kDa domain of hsp90. J Biol Chem *273*, 18007-18010.

Young, J. C., Schneider, C., and Hartl, F. U. (1997). In vitro evidence that hsp90 contains two independent chaperone sites. FEBS Lett *418*, 139-143.

Zabarovsky, E. R., and Winberg, G. (1990). High efficiency electroporation of ligated DNA into bacteria. Nucleic Acids Res *18*, 5912.

Zhao, Q., Boschelli, F., Caplan, A. J., and Arndt, K. T. (2004). Identification of a conserved sequence motif that promotes Cdc37 and Cyclin D1 binding to Cdk4. J Biol Chem.

Zhou, G., Bao, Z. Q., and Dixon, J. E. (1995). Components of a new human protein kinase signal transduction pathway. J Biol Chem *270*, 12665-12669.

Zu, T., Verna, J., and Ballester, R. (2001). Mutations in WSC genes for putative stress receptors result in sensitivity to multiple stress conditions and impairment of Rlm1-dependent gene expression in *Saccharomyces cerevisiae*. Mol Genet Genomics *266*, 142-155.